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USE OF VARIOUS FUNCTIONS TO ANALYSE THE FERTILISER RESPONSES OF MAIZE (*Zea mays* L.) HYBRIDS IN LONG-TERM EXPERIMENTS

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The effect of various fertiliser treatments on the yield of maize hybrids was studied on the basis of 26 years of data obtained in a long-term bifactorial split-plot experiment set up in 1967. The seven treatments (NPK ratio 2:1:1) applied were as follows (rates per hectare): 1. Control (no fertiliser), 2. 100 kg NPK, 3. 200 kg NPK, 4. 300 kg NPK, 5. 400 kg NPK, 6. 600 kg NPK, 7. 800 kg NPK. The maize was grown with the conventional cultivation techniques in continuous cropping.

The results of analyses carried out with three different methods (analysis of variance, cumulative yield analysis and regression analysis) all indicated that under the given conditions the yield of maize hybrids was highest at an NPK fertiliser rate of 200–400 kg ha^{-1}. The effect of fertilisation on the maize yield was significant in 21 of the 26 years. Combined analysis of variance for the years showed that the year effect (quantity of rainfall) had the greatest effect on the maize yield, but although the year effect had a fundamental effect on the yield level it did not influence the fertiliser response pattern.

The fertiliser responses of the maize hybrids were described by fitting four types of functions (quadratic, square root, inverse exponential, linear-plateau) to the yield data. It was found that when selecting the best function a consideration of the regression deviations (measured yield – calculated yield) was just as important as the coefficient of determination (R^2). In 12 of the 26 years the fitting of the quadratic function was not significant and overestimated the fertilisation optimum. The fertiliser response curve generally has a broad maximum which is far better described by the square root function than by the quadratic. If the fertiliser response pattern includes a depressive phase, a square root function should definitely be used in place of the quadratic function. If the maximum of the response surface forms a plateau (as opposed to a maximum point) a linear-plateau function or an inverse exponential function can be recommended. In the present work the linear-plateau function gave the best results.

Key words: maize, optimal fertiliser rate, response model, long-term experiment

Introduction

The analysis of the fertiliser responses of crops and the determination of optimum fertiliser rates requires the fitting of various functions to the yield data from fertilisation rate experiments. Research on the functions best suited for the description of fertiliser responses has been underway for over a century. Liebig's famous Law of the Minimum can be regarded mathematically as a "linear response and plateau" model (Paris, 1992). Mitscherlich (1909) applied an exponential function to describe yield curves. Although Mitscherlich's function has been said to be in opposition to Liebig's hypothesis, there is in fact no contradiction between them (Paris, 1992). The extension of the analysis of plant responses to more than one nutrient can be attributed to Baule (1918), who proposed a multiplicative relationship between nutrients.

Despite the work of Baule (1918) the fitting of functions to yield responses involving two or more nutrients remained an extremely difficult proposition until Heady and Pesek (1954) recommended the use of polynomial functions (quadratic, square root, etc.). These models give a good fit to experimental data and the quadratic function in particular has been widely used to describe the fertiliser responses of crops, partly because of its simplicity.

Over the following decades, however, it gradually became clear that, despite the good fit of these polynomial functions, as measured by the coefficient of determination (R^2), they seriously overestimated the optimum rate of fertiliser (Anderson and Nelson, 1975). By the end of the 1970s there was no longer any doubt that polynomials were not always a satisfactory way of making fertiliser recommendations. This, together with environmental concerns, demonstrated the need to re-evaluate the analysis of plant responses (Liebhardt et al., 1989).

In many cases environmental or agronomic factors may induce a ceiling effect on yield, in which case the maximum of the response surface will resemble a plateau rather than a maximum point. Both the crop and the response type have an influence on the response pattern, including the existence of a plateau and where it begins. Anderson and Nelson (1975) were of the opinion that no single continuous model could be found, capable of fitting all types of response and giving an accurate estimation of optimum rates. These authors elaborated a family of linear-plateau functions, consisting of two or more intersecting straight lines, rather than a single function. The term linear-plateau function reflects the fact there is a region of linear response (possibly involving more than one slope) and a plateau.

Cerrato and Blackmer (1990) recommended a quadratic-plateau function to replace the quadratic function. A few years later Overman et al. (1994) described the fertiliser responses of crops using an inverse exponential model, which can be regarded as a modified Mitscherlich function. A modified form of the tanh (N) function (hyperbolic tangent) was reported by Olness et al. (1995), while a general tanh (N) model (Hotelling, 1927) was cited by Richards (1969).

For many decades crop producers evaluated empirical models only on the basis of the coefficient of determination (R^2), but Anderson and Nelson (1975) suggested that this was not the best criterion for estimating optimum fertiliser rates. Cerrato and Blackmer (1990) pointed out that the coefficient of determination was not a relevant statistic for the selection of the best model and that other, more rigorous criteria should be relied on for choosing the appropriate specification.

Results achieved so far make it clear that the selection of the best function requires more attention than it was given in the past. The database for the present research consisted of a 26-year data series from a long-term fertilisation experiment set up in 1967. The aim of the work was to evaluate treatment effects using analysis of variance and the cumulative yield difference method, and to compare the applicability of four different functions (quadratic, square root, inverse exponential and linear-plateau) for analysing the fertiliser responses of maize hybrids.

Materials and methods

Experimental treatments

The two-factor, split-plot fertilisation experiment was set up by Béla Győrffy and his colleagues in the institute nursery in Martonvásár in 1967. The area is slightly eroded and the soil, a chernozem with forest residues, is a humous loam, slightly acidic in the ploughed layer, poor in phosphorus, but well supplied with potassium.

The fertiliser treatments in the main plot were arranged as a random block with four replications, with a plot size of 8 × 7 m = 56 m^2. The NPK fertiliser (NPK ratio 2:1:1) was applied at the following rates (kg ha^{-1}): 1. Control (without fertiliser), 2. 100 kg NPK, 3. 200 kg NPK, 4. 300 kg NPK, 5. 400 kg NPK, 6. 600 kg NPK, 7. 800 kg NPK. The maize hybrids forming the sub-plots generally consisted of three mid-season hybrids, replaced by others every 4–6 years. In each sub-plot there were two rows of each hybrid, separated from each other by buffer rows. The aim of the experiments was to determine the effect of high NPK fertiliser rates on the yield of maize hybrids and to analyse the fertiliser responses of maize inbred lines over a short period (1976–1982). The hybrids were grown in a monoculture with standard agronomical practices, and the fertiliser treatments remained unchanged throughout the experiment.

The fertiliser responses of the hybrids were evaluated on the basis of 26 years of data. The results of experiments on maize inbred lines between 1976 and 1982 and the data for 1990, when the crop was completely destroyed by severe drought, were omitted from the analysis.

Analysis of variance (ANOVA)

In the first step differences in yield between the experimental treatments were subjected to two-factor ANOVA (23 years). Three years, when only one maize hybrid was tested, were evaluated as a single-factor random block design. In the second step the main effects of the experimental treatments and the interactions between the treatments were analysed using combined two-factor ANOVA on the data of all the years, as described by Sváb (1981) and Gomez and Gomez (1984). ANOVA was also used to investigate changes in the regression deviations when fitting various functions.

Cumulative yield analysis

The effect of the experimental treatments on the yield was first analysed using the cumulative method elaborated for long-term experiments (Sváb, 1981), in which annual and cumulative yield differences between the treatments and the basic treatment (100 kg ha^{-1}) are calculated each year. The cumulative yield differences indicate the total yield difference between the given treatment and a basic treatment in the tth year. This comparison to the basic treatment is required to eliminate the year effect, but the treatment × year interaction is not eliminated.

Regression analysis

Based on results in the literature and our own research, four functions (quadratic, square root, inverse exponential and linear-plateau) were fitted to the annual yield data, using the NLIN (non-linear) and GLM (general linear model) procedures of the SPSS program. The maize hybrids were evaluated together in the regression analysis, as ANOVA revealed that their fertiliser responses did not generally differ.

The quadratic and square root functions widely used to characterise the fertiliser response of maize hybrids are given in Equations (1) and (2):

$$Y = a + bx + cx^2 \tag{1}$$

$$Y = a + bx + cx^{1/2} \tag{2}$$

where Y is the grain yield (t ha^{-1}), X is the fertiliser rate (kg ha^{-1}), a is the intercept, and b and c are the linear and quadratic regression coefficients.

The inverse exponential function reported by Overman et al. (1994), also known as the modified Mitscherlich function, can be expressed as:

$$Y = m(1+e^{-(cx-b)})^{-1} \tag{3}$$

where Y is the yield (t ha^{-1}), m is the maximum yield, b is an intercept parameter for yield, X is the fertiliser rate (kg ha^{-1}) and c is the fertiliser response coefficient.

The linear-plateau model proposed by Anderson and Nelson (1975) can be described by Equations (4) and (5):

$$Y = a + bX \text{ if } X < C \tag{4}$$

$$Y = P \text{ if } X \geq C \tag{5}$$

where Y is the grain yield (t ha^{-1}), X is the fertiliser rate (kg ha^{-1}), a is the regression constant, b is the regression coefficient, C is the critical fertiliser rate (which occurs at the intersection of the linear regression and the plateau lines) and P is the plateau yield (C and P are constants obtained by fitting the model to the data).

Each function was fitted to 26 data sets. The residual deviations were analysed as reported by Bullock and Bullock (1994) and Olness et al. (1998). Normality was analysed using the Kolmogorov–Smirnov and Shapiro–Wilk tests (SPSS for Windows, 1999), while the paired t-test was used to determine the significance of regression deviations between the functions (Bullock and Bullock, 1994). The deviation from regression is illustrated in the form of dot plots and box plots. The box plots illustrate five points of the distribution: the lowest value, the lower quartile, the median, the upper quartile and the highest value. Outlying values are designated by circles.

The maximum yields predicted by the quadratic and square root functions were calculated using the formalae $Y_{max} = a - b^2/4c$ and $Y_{max} = c^2/4b^2$. In the case of the linear-plateau and inverse exponential functions, the plateau yield (P) and the maximum value (m), respectively, were taken to represent the maximum yield. For these two functions the optimum fertiliser rate was determined by solving the equations.

The biometric analysis of the data was carried out using the MSTAT-C (1991) and SPSS for Windows (1999) programs.

Results

Evaluation of yield results using ANOVA and cumulative yield analysis

The effect of fertilisation on the yields of maize hybrids was evaluated using two-factor (in 23 years) or single-factor (in 3 years) analysis of variance (Table 1). In 21 of the 26 years this effect was found to be significant. Apart from the first year of the experiment, non-significant fertiliser effects were generally observed in dry years, when water deficiency limited the yield to such an extent that the yield was unable to respond to fertilisation. With the exception of three years the hybrid effect was significant, but this was caused by the diverse yield levels of the hybrids rather than by differences in fertiliser response. In 18 of the 23 years there was no significant fertilisation × hybrid interaction, indicating that the fertiliser responses of the maize hybrids did not differ under the given experimental conditions. The results of combined ANOVA (data not shown) revealed that the year had the greatest effect on the maize yield (MS value: 276.1), followed by the fertiliser effect (MS value: 164.9), while the hybrid effect was substantially smaller (MS value: 18.1). The most important of the interactions was the year × hybrid interaction (MS value: 8.1). The results of combined ANOVA showed that at NPK rates in excess of 300 kg ha^{-1} additional fertiliser did not have a significant effect on the yield and that the maximum fertilisation effect could be represented by a plateau rather than by a maximum point. Averaged over 23 years the maize yields (t ha^{-1}) in the various fertiliser treatments were as follows: 1: 4.238d, 2: 5.520c, 3: 6.466a, 4: 6.316ab, 5: 6.503a, 6: 6.425ab, 7: 6.226b (treatments denoted by the same letters did not differ significantly on the basis of Duncan's Multiple Range Test).

The annual yield response data were used to illustrate the cumulative effect of mineral fertiliser treatment on the maize grain yield (Fig. 1). The 100 kg ha^{-1} fertiliser rate was taken as the basic treatment and the cumulative yield differences compared to this treatment represent the effects of different fertiliser rates. It can be clearly seen that the unfertilised control led to increasing yield losses, as the curve sloped downwards, exhibiting a total loss of 35.84 t ha^{-1} compared to the basic treatment after 26 years. There was very little difference between the various fertilisation treatments in the first 6–8 years, providing a clear illustration of the fact that meaningful information on the effect of fertilisation treatments can only be obtained from long-term experiments carried out over several decades.

The cumulative long-term effects of the fertiliser rates could be divided into three groups (Fig. 1). The greatest yield surplus compared with the basic treatment was observed at NPK rates of 200 and 400 kg ha^{-1} (25.89 and 25.53 t ha^{-1}, respectively). For the second group (300 and 600 kg ha^{-1} NPK) the yield surplus compared with the basic treatment was lower (19.49 and 21.27 t ha^{-1}, respectively), while the surplus was considerably less for the 800 kg ha^{-1} NPK rate (15.07 t ha^{-1}).

Table 1

Results of analysis of variance for each year of the long-term experiment
(d.f. values for: replication: 3; NPK rate (A): 6; error: (a): 18; hybrid (B): 2; A × B: 12; error (b): 42)

Year	Source of variation						CV%
	Replication	NPK rate (A)	Error (a)	Hybrid (B)	A × B	Error (b)	
			MS values				
1967	2.40	0.58^{NS}	0.32	–	–	–	9.63
1968	2.35	2.20^{**}	0.32	2.87^{**}	0.27^{NS}	0.33	11.11
1969	0.25	20.84^{***}	1.44	7.55^{***}	0.43^{NS}	0.23	6.32
1970	0.34	19.36^{***}	0.58	5.57^{***}	0.17^{NS}	0.13	7.36
1971	6.27	5.03^{*}	1.38	4.93^{**}	0.17^{NS}	0.23	8.46
1972	0.57	3.04^{**}	0.29	5.82^{**}	0.29^{NS}	0.22	7.14
1973	0.98	2.66^{**}	0.36	1.09^{*}	0.45^{NS}	0.23	8.10
1974	0.18	2.16^{**}	0.20	0.10^{NS}	0.25^{*}	0.10	5.58
1975	0.28	25.22^{***}	0.67	42.25^{***}	1.33^{***}	0.23	6.05
1983	2.75	2.12^{**}	0.36	–	–	–	17.45
1984	4.39	15.58	1.55	20.41^{***}	1.26^{NS}	0.79	13.81
1985	1.93	11.54^{***}	0.59	7.26^{***}	0.11^{NS}	0.36	9.25
1986	3.02	25.85^{**}	0.91	192.48^{**}	4.12^{**}	1.02	8.72
1987	4.41	17.56^{***}	1.71	16.26^{***}	0.39^{NS}	0.47	10.16
1988	2.89	1.83^{NS}	1.52	1.36^{**}	0.78^{**}	0.23	19.03
1989	1.68	12.29^{***}	0.75	18.64^{***}	1.00^{**}	0.29	9.83
1991	3.41	14.17^{***}	1.70	15.82^{***}	0.30^{NS}	0.68	10.01
1992	3.69	5.96^{*}	1.88	2.22^{NS}	0.71^{NS}	1.05	23.66
1993	2.20	4.05^{NS}	2.95	1.71^{NS}	0.41^{NS}	0.99	24.25
1994	7.43	1.90^{NS}	1.71	1.50^{NS}	0.44^{NS}	0.60	26.27
1995	2.26	6.02^{**}	1.32	5.12^{**}	0.53^{NS}	0.66	11.41
1996	13.43	18.03^{***}	1.69	5.73^{*}	2.85^{NS}	1.55	20.97
1997	0.58	4.68^{**}	1.12	–	–	–	15.36
1998	3.03	16.45^{**}	3.75	5.48^{*}	1.78^{NS}	1.12	14.64
1999	13.30	60.22^{***}	1.34	21.50^{***}	1.77^{NS}	1.23	11.03
2000	4.71	5.91^{NS}	3.05	7.06^{***}	0.43^{NS}	0.62	15.59

^{NS} = non-significant, ***, **, * = significant at the P = 0.1%, 1% and 5% level, respectively

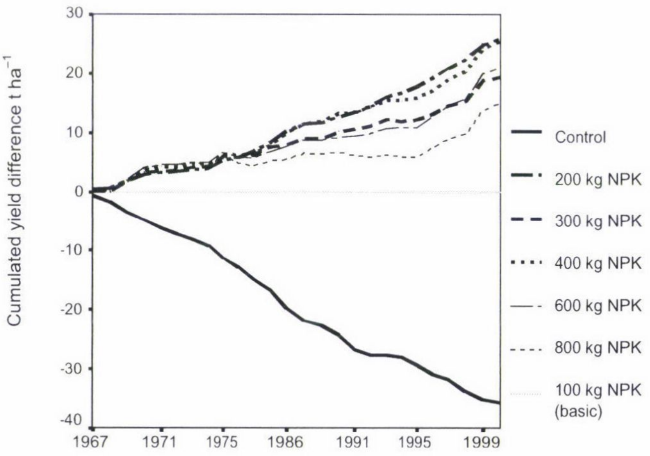

Fig. 1. Cumulative effect of fertilisation on maize grain yield in a long-term experiment (1967–2000)

Analysis of the fertiliser responses of maize hybrids using regression analysis

The fertiliser responses of the hybrids were described by fitting four types of functions to the experimental yield data (quadratic, square root, inverse exponential, linear-plateau). Table 2 lists the coefficients of determination (R^2) for the various functions describing the relationships between the fertiliser rates and the maize yields in the given years. The level of significance based on the F test is also indicated. It is clear that the worst fit was obtained with the quadratic function, where R^2 was non-significant in 12 of the 26 years, and significant at the 5% level in 10 years and at the 1% level in four. The fitting of the square root function was significant at the 0.1% level every year. The R^2 values for the inverse exponential and linear-plateau functions were similar in magnitude and indicated that fitting was significant at the 0.1% level in all but one or three years, respectively.

Table 2

Coefficients of determination for models describing relationships between NPK rate and maize yield in the experimental years[†]

Year	R^2 values			
	Quadratic	Square root	Inverse exponential	Linear-plateau
1967	0.665^{NS}	0.358***	0.866***	0.958***
1968	0.649^{NS}	0.919***	0.976***	0.977***
1969	0.913**	0.966***	0.991***	0.993***
1970	0.965**	0.976***	0.995***	0.967***
1971	0.820*	0.978***	0.978***	0.976***
1972	0.664^{NS}	0.962***	0.958***	0.959***
1973	0.875*	0.997***	0.994***	0.981***
1974	0.759^{NS}	0.986***	0.745***	0.745***
1975	0.872*	0.970***	0.992***	0.993***
1983	0.697^{NS}	0.958***	0.333**	0.573**
1984	0.580^{NS}	0.900***	0.756***	0.900***
1985	0.845*	0.946***	0.947***	0.973***
1986	0.739^{NS}	0.874***	0.820***	0.869***
1987	0.851*	0.962***	0.971***	0.975***
1988	0.634^{NS}	0.880***	0.718***	0.719**
1989	0.819*	0.917***	0.846***	0.864***
1991	0.705^{NS}	0.971***	0.848***	0.849***
1992	0.831*	0.808***	0.606***	0.747**
1993	0.585^{NS}	0.498***	0.413***	0.683***
1994	0.256^{NS}	0.313***	0.157***	0.665***
1995	0.500^{NS}	0.797***	0.695***	0.930***
1996	0.869*	0.926***	0.930***	0.936***
1997	0.916**	0.876***	0.928***	0.962***
1998	0.811*	0.936***	0.937***	0.970***
1999	0.975**	0.921***	0.984***	0.984***
2000	0.874*	0.851***	0.888***	0.889***

[†] Significance levels were determined using the F-test. Levels of significance: ^{NS} = non-significant, ***, **, * = significant at the P = 0.1%, 1% and 5% level, respectively

As seen from the data in Table 3, the functions all predicted similar maximum yields, though every year the highest value (7.02 t ha^{-1} averaged over 26 years) was obtained using the quadratic function. This was slightly in excess of the highest yield actually recorded on the average of 26 years (6.76 t ha^{-1}). The maximum yield determined by fitting the square root function (6.80 t ha^{-1}) exhibited the least deviation to the measured data, while the inverse exponential and linear-plateau functions gave similar yield maximums (6.65 and 6.56 t ha^{-1}, averaged over 26 years).

Table 4 presents the optimum NPK fertiliser rates predicted by the functions for each year. Averaged over 26 years the optimum was 490 kg NPK ha^{-1} according to the quadratic function, more than twice that predicted using the linear-plateau model (234 kg ha^{-1} NPK) and 66 % greater than the optimum predicted by the inverse exponential function (295 kg ha^{-1}). The optimum NPK rate predicted by the square root function was 418 kg ha^{-1} averaged over 23 years. In three years (1970, 1999, 2000) there was no decline in the measured yield data, so the optimum of the square root function would have been an extrapolated value, for which there is no empirical evidence. These data were thus omitted from the analysis.

Table 3

Maximum maize yields predicted by various functions in the experimental years

Year	Predicted maximum yield (t ha^{-1})			
	Quadratic	Square root	Inverse exponential	Linear-plateau
1967	6.18	6.02	6.02	5.97
1968	5.50	5.39	5.30	5.28
1969	8.96	8.55	8.52	8.43
1970	6.25	6.18	6.00	5.94
1971	6.22	6.02	5.94	5.93
1972	7.06	6.95	6.83	6.81
1973	6.41	6.25	6.21	6.21
1974	5.99	5.92	5.73	5.73
1975	9.40	8.95	8.87	8.80
1983	4.00	4.09	3.59	3.32
1984	7.25	7.13	6.78	6.57
1985	7.66	7.23	7.12	7.06
1986	13.28	12.71	12.36	12.17
1987	7.92	7.50	7.41	7.40
1988	2.83	2.78	2.62	2.63
1989	6.46	6.10	5.94	5.93
1991	9.22	8.99	8.61	8.62
1992	5.04	4.81	4.61	4.52
1993	4.60	4.41	4.33	4.24
1994	3.14	3.13	3.00	2.85
1995	7.65	7.57	7.36	7.20
1996	7.11	6.82	6.69	6.57
1997	7.98	7.61	7.65	7.52
1998	8.33	7.92	7.81	7.72
1999	12.31	12.17	11.97	11.71
2000	5.73	5.63	5.59	5.45

Table 4

Optimum NPK fertiliser rates predicted by various functions in the experimental years

Year	Predicted optimum NPK rate (kg ha^{-1})			
	Quadratic	Square root	Inverse exponential	Linear-plateau
1967	510	590	230	200
1968	530	450	190	200
1969	550	640	350	300
1970	610	–	430	350
1971	540	530	250	200
1972	480	360	200	200
1973	520	500	210	200
1974	420	270	100	150
1975	540	590	300	250
1983	350	200	100	280
1984	430	280	170	250
1985	490	420	250	200
1986	470	360	230	250
1987	510	480	310	200
1988	440	320	170	170
1989	460	350	260	180
1991	440	300	150	150
1992	430	290	230	250
1993	430	300	250	250
1994	380	220	110	250
1995	440	290	200	250
1996	570	760	390	250
1997	530	670	430	250
1998	500	440	280	250
1999	590	–	620	350
2000	570	–	430	250

The extent to which the various functions fitted the 26 years of experimental data can be clearly seen in Figure 2. Both the inverse exponential and the linear-plateau functions gave a good fit, predicting lower yields on unfertilised plots than the quadratic function and larger yield responses, close to those actually obtained, up to NPK rates of 200–250 kg NPK. The quadratic function continued to predict unrealistic yield increases up to rates of 400–600 kg NPK, thus greatly overestimating the optimum rate. The square root function gave a very good fit to the data. This figure clearly illustrates the need to examine at least 5–8 fertiliser rates for several years at several locations if the analysis of fertiliser responses and the determination of optimum fertiliser rates are to be reliable.

Analysis of deviations from regression (residuals)

The deviation from regression, or residuals (measured yield – yield predicted by the model) obtained when fitting various functions is illustrated as a dot plot in Figure 3 and as a box plot in Figure 4. On the residual plot, points above the horizontal line (residual = 0) indicate where observed yield data were higher than the predicted values, and those below the line indicate where observed data were lower than the predicted values. The figures confirm the results of ANOVA, indicating that the deviations from regression (residuals) differed for each fertiliser rate for any given function. In the case of the quadratic function the mean deviation from regression was greatest at the 200 kg ha^{-1} NPK rate (0.567), followed by the control treatment (–0.365) and the 600 kg ha^{-1} NPK rate (–0.316), while the smallest value was obtained at 300 kg ha^{-1} (–0.126). For the square root function the deviation from regression was greatest at 200 kg ha^{-1} (0.315) and 100 kg ha^{-1} (–0.292) and smallest at 600 kg ha^{-1} (–0.0191) and 800 kg ha^{-1} (–0.252). The greatest deviation from regression for the inverse exponential function was observed at a rate of 200 kg ha^{-1} (0.383), followed by 800 kg ha^{-1} (–0.294), with the lowest value at 300 kg ha^{-1} (–0.0339). For the linear-plateau function the highest values for the deviation from regression were recorded at 800 kg ha^{-1} (–0.224) and 400 kg ha^{-1} (0.181) and the lowest values at 300 kg ha^{-1} (–0.0281).

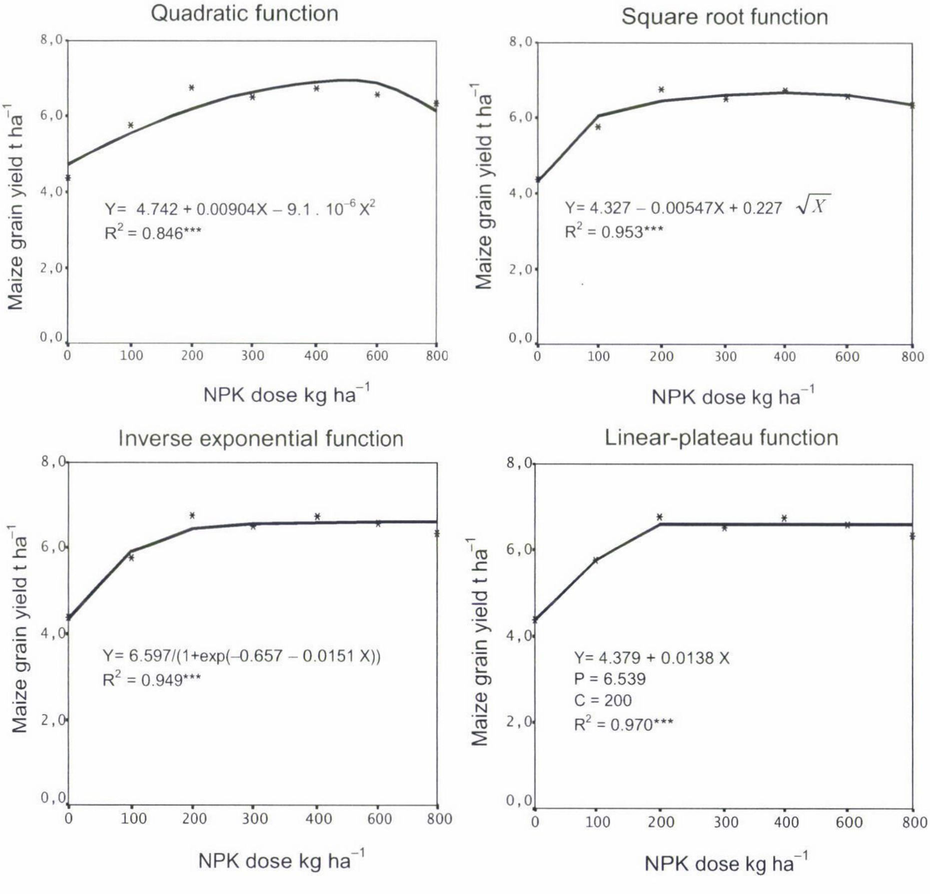

Fig. 2. Prediction of changes in maize grain yield as a function of fertiliser dose by fitting four different functions (average of 26 years). Asterisk indicates measured yields

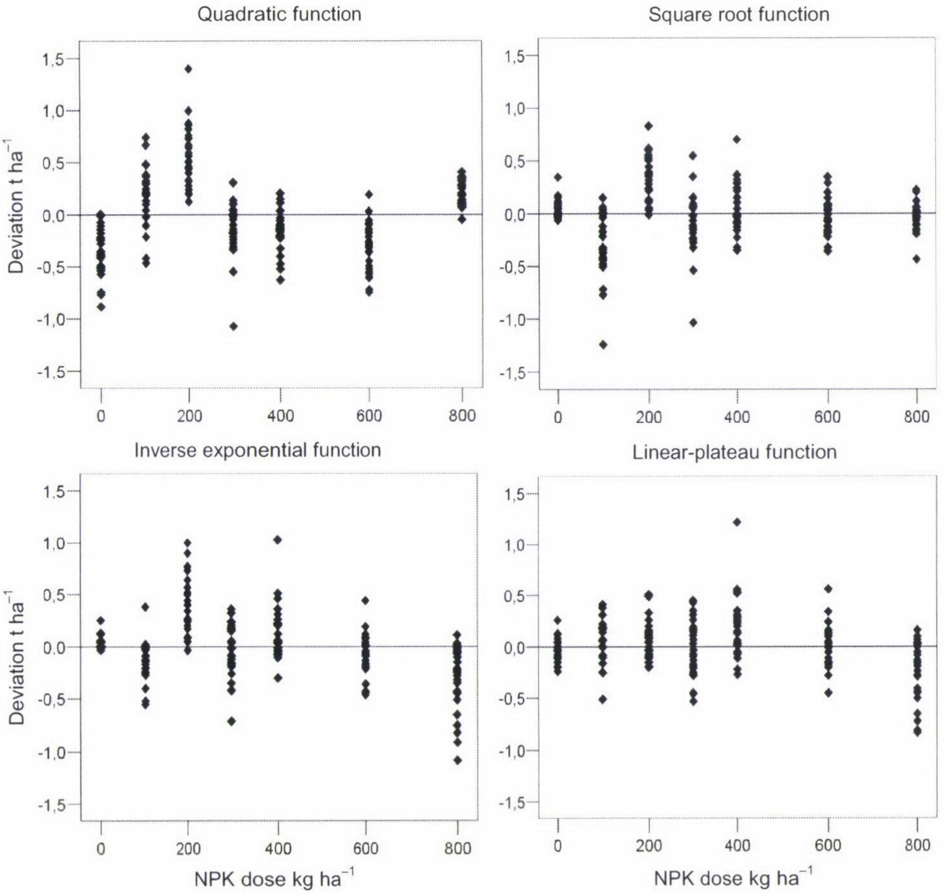

Fig. 3. Deviation from regression (measured yield – predicted yield) when fitting various functions. Each point is the treatment mean for a given year (n = 26)

In most cases the deviation (residual) from the regression functions at various fertiliser rates exhibited a random distribution, except in the case of the quadratic model, where the values of these deviations for different fertiliser rates followed a sinusoidal pattern, in agreement with the results reported by Bullock and Bullock (1994) and Olness et al. (1998). However, the analysis of normality using the Kolmogorov–Smirnov and Shapiro–Wilk tests indicated that the residuals obtained from the quadratic function had standard normal distribution.

The paired t-test was used to determine whether the deviations from regression were significantly different for the four functions (n = 182). The differences between the squares of the regression deviations (residuals) [e.g. difference = (quadratic deviations)^2 – (inverse exponential deviations)^2] proved to be significant for four function pairs. The greatest difference was found between the quadratic and square root functions (mean difference: 0.076, t = 4.66, P = 0.1%) and the quadratic and linear-plateau functions (mean difference:

0.0864, t = 4.10, P = 1%), followed by the difference between the quadratic and inverse exponential functions (mean difference: 0.0578, t = 3.38, P = 1%) and the inverse exponential and linear-plateau functions (mean difference: 0.0285, t = 2.66, P = 5%). No significant difference was found between the regression deviations of the square root and linear-plateau functions or between those of the square root and inverse exponential functions.

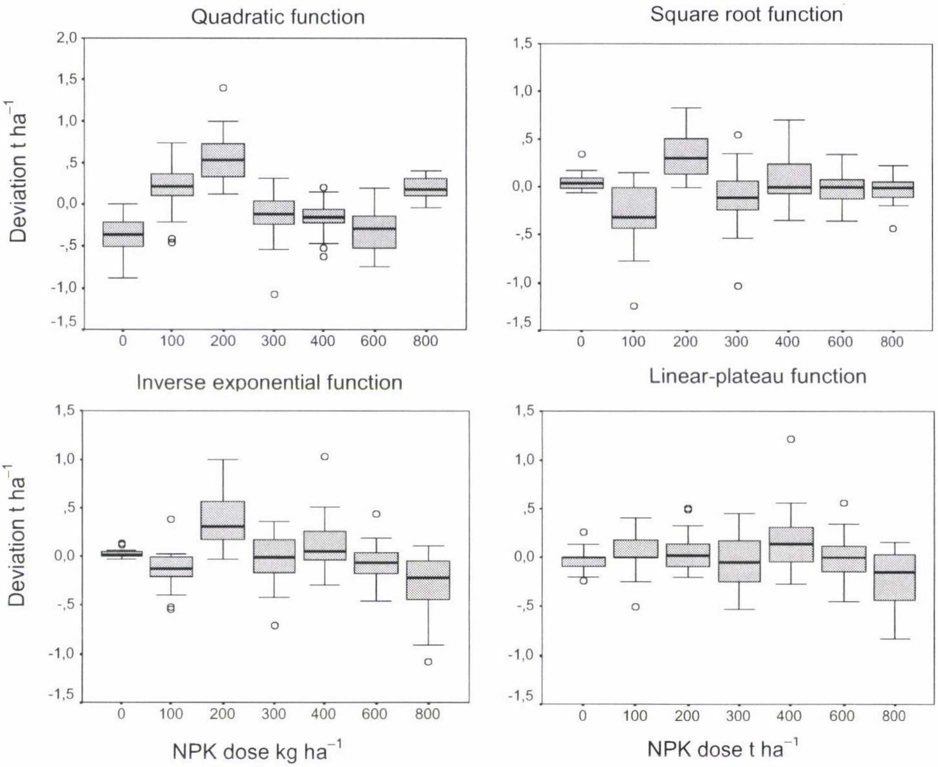

Fig. 4. Box plot of deviation from regression (measured yield – predicted yield) when fitting various functions. Box plots show the distribution of the values by medians (central line), lower (25%) and upper (75%) quartiles (box) and ranges (n = 26)

Discussion

The results of analysis using three different methods (ANOVA, cumulative yield analysis and regression analysis) concurred in showing that maize hybrids gave their highest yields at NPK fertiliser rates of 200–400 kg ha^{-1}. Combined analysis of variance showed that the year (particularly the rainfall quantity) had the greatest influence on yield. In the experimental years the maize yield ranged from 2.53 to 10.03 t ha^{-1}, averaged over fertiliser treatments and hybrids, with a median value of 5.77 t ha^{-1}. Except in extremely dry years, the fertiliser effect was significant every year and could be attributed chiefly to the effect of N fertiliser. The year effect fundamentally determined the yield level, while having no influence on the fertiliser response pattern.

The results make it quite clear that the choice of function deserves far greater attention than it has previously been afforded. In agreement with the findings of Anderson and Nelson (1975) and Cerrato and Blackmer (1990), the present work demonstrated that the coefficient of determination (R^2) is not a sufficient criterion in itself for judging the goodness of function fitting. Although it was clear from the R^2 value that, of the four functions examined, the quadratic function did not always give a satisfactory fit, no difference could be found between the other three functions on the basis of this value. As previously reported by Cerrato and Blackmer (1990), Bullock and Bullock (1994) and Olness et al. (1998), the present work also indicated that, in addition to the coefficient of determination, the analysis of residual deviation (measured yield – predicted yield) is an essential criterion for the choice of function.

So which functions should be fitted? For the sake of simplicity, as regards both interpretation and computing, quadratic functions are generally used for the determination of maximum yield. However, as demonstrated in the present research, the quadratic function is not ideal for determining maximum yield and the relevant optimum fertiliser rate, as it may result in the misleading conclusion that the yield achieved at the highest rate applied in the experiment is close to maximum and that a further increase in the rate would lead to yield depression. This disadvantage of the quadratic function stems from the fact that the function has a very pronounced curve close to the maximum. The fertiliser effect curve is generally characterised by a broad maximum region, which is better described by a square root function. The maximum calculated from this function is generally an extrapolated value, if there is no yield decline. This is a reasonable assumption, as in such cases no empirical evidence is available on the maximum.

In many cases environmental or agronomical factors may induce a ceiling effect on the yield, in which case the real maximum of the response surface is a plateau rather than a maximum point. For such response types either the family of linear-plateau functions elaborated by Anderson and Nelson (1975) or the inverse exponential function (Overman et al., 1994) can be recommended. In the present work the linear-plateau function gave the best results with regard to both the goodness of fit and the values obtained for maximum yield and optimum fertiliser rate. At the same time, Cerrato and Blackmer (1990) pointed out that the linear-plateau model may over-estimate the yield in the section of the response curve containing the fertiliser optimum. The over-estimation of the yield could result in the under-estimation of the optimum fertiliser rate.

Acknowledgements

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References

Anderson, R. L., Nelson, L. A. (1975): A family of models involving intersecting straight lines and concomitant experimental design useful in evaluating response to fertilizer nutrients. *Biometrics,* **31,** 303–318.

Baule, B. (1918): Zu Mitscherlichs Gesetz der physiologischen Beziehungen. *Landwirtschaftliche Jahrbücher,* **57,** 363–385.

Bullock, D. G., Bullock, D. S. (1994): Quadratic and quadratic-plus-plateau models for predicting optimal nitrogen rate of corn: a comparison. *Agron. J.,* **86,** 191–195.

Cerrato, M. E., Blackmer, A. M. (1990): Comparison of models for describing corn yield response to nitrogen fertilizer. *Agron. J.,* **82,** 138–143.

Gomez, K. A., Gomez, A. A. (1984): *Statistical Procedures for Agricultural Research.* John Wiley and Sons, New York.

Heady, E. O., Pesek, J. T. (1954): A fertilizer production surface. *J. Farm Econ.,* **36,** 466–482.

Hotelling, H. (1927): Differential equations subject to error, and population estimates. *J. Am. Stat. Assoc.,* **22,** 283–314. (Cit. F. J. Richards, 1969)

Liebhardt, W. C., Andrews, R. W., Culik, M. N., Harwood, R. R., Janke, R. R., Radke, J. K., Rieger-Schwartz, S. L. (1989): Crop production during conversion from conventional to low-input methods. *Agron. J.,* **81,** 150–159.

Mitscherlich, E. A. (1909): Das Gesetz des Minimums und das Gesetz des abnehmenden Bodenertrages. *Landwirtschaftliche Jahrbücher,* **38,** 537–552.

MSTAT-C (1991): *A Microcomputer Program for the Design, Management, and Analysis of Agronomic Research Experiments.* MSTAT Development Team, Michigan State University.

Olness, A., Evans, S. D., Moncrief, J. F. (1995): Maize grain yield response to tillage and fertilizer nitrogen rates on a Tara silt loam. *J. Agron. Crop Sci.,* **174,** 273–285.

Olness, A., Evans, S. D., Alderfer, R. (1998): Calculation of optimal fertilizer rates: a comparison of three response models. *J. Agron. Crop Sci.,* **180,** 215–222.

Overman, A. R., Wilkinson, S. R., Wilson, D. M. (1994): An extended model of forage grass response to applied nitrogen. *Agron. J.,* **86,** 617–620.

Paris, Q. (1992): The return of von Liebig's "Law of the Minimum". *Agron. J.,* **84,** 1040–1046.

Richards, F. J. (1969): The quantitative analysis of growth. pp. 3–76. In: Steward, F. C. (ed.), *Plant Physiology, A Treatise, Vol. V-A, Analysis of Growth: Behavior of Plants and their Organs.* Academic Press, New York.

SPSS for Windows (1999): *User's Guide. Release 10.0.* SPSS Inc., Chicago.

Sváb, J. (1981): *Biometriai módszerek a kutatásban.* (Biometric Methods for Research.) Mezőgazdasági Kiadó, Budapest.

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IDENTIFICATION OF CHROMOSOME REGIONS INVOLVED IN THE GENETIC REGULATION OF TILLERING IN BARLEY (*Hordeum vulgare* L.)

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Tillering ability is a complex trait, the development of which is influenced by both environmental factors and complex genetic regulation. In the present experiments this complex regulation was dissected into its various components in an effort to separate the effect on tillering of major genes influencing ontogeny from that of other genomic factors. The tillering rate of a facultative × winter barley mapping population was examined in the field after autumn and spring sowing. The vernalisation sensitivity gene *Vrn-H2* exerted a considerable influence on tillering in spring-sown barley. In addition to the major genes, QTL analysis revealed two chromosome regions (1HS and 3HL) with a significant influence on the extent of tillering. Neither of these regions were involved in the regulation of heading date, and their effect on tillering was the most intense at the beginning of ontogeny, gradually declining as the influence of the *Vrn-H2* gene increased. The function of the *Vrn-H2* locus in the regulation of tillering is manifested partly through a direct effect on the transition from the vegetative to the generative phase and partly indirectly via epistatic regulation of other chromosome regions influencing tillering.

Key words: barley, genetic regulation, QTL, tillering, *Vrn-H2*

Introduction

The tillering ability of cereals, and thus the number of productive side-shoots, is one of the main components of yield potential. Tillering vigour in early stages of plant development, i.e. how soon tillering begins and at what rate new tillers are formed, may also be important for biofarming, since intensively tillering genotypes cover the ground more quickly, thus giving better weed suppression. The extent of tillering is considerably influenced by environmental factors (rainfall, nutrient supplies, temperature, etc.), but the genotype also plays a significant role. Among the factors involved in genomic determination, genes regulating ontogeny have a substantial influence on tillering ability; until the plant is exposed to the environmental stimuli (daylength, cold effect) required for the initiation of the generative phase, it remains in the vegetative phase, which automatically leads to a higher rate of tillering. This is particularly noticeable in winter growth types, which remain in the vegetative phase with intense tillering if they are not exposed to vernalisation treatment. A similar effect may be produced by the failure to satisfy the daylength requirement in genotypes sensitive to daylength. Prior to shooting, the majority of the assimilates are utilised for the formation of tillers, while after shooting the assimilates are divided between shoot growth and the formation of new tillers.

Numerous experiments have confirmed that vernalisation requirement and daylength sensitivity have a pleiotropic effect on tillering ability and also on certain yield components (Snape et al., 1985; Laurie et al., 1995; Worland 1996; Kato et al., 2000). Investigations on wild barley series indicated that the effect of vernalisation requirement on tillering was daylength-dependent, confirming the complex, combined role of these two factors in tillering, too (Karsai et al., 2005a). The effect of individual vernalisation and daylength sensitivity genes on tillering was demonstrated by means of QTL analysis (Laurie et al., 1994; Karsai et al., 1999; Kato et al., 2000).

Up till now, two major genes have been identified as being involved in the vernalisation regulation pathway in barley. The *Vrn-H1* gene (Danyluk et al., 2003; Yan et al., 2003; von Zitzewitz et al., 2005) on the long arm of chromosome 5H, which stimulates the heading date, is regulated by the *Vrn-H2* gene on chromosome 4H. The latter is dominant for winter growth type and codes a repressor protein, the production of which ceases as the result of vernalisation treatment (Yan et al., 2004; Dubcovsky et al., 2005; von Zitzewitz et al., 2005). The effect of both *Vrn-H2* and *Vrn-H1* on the heading date has been confirmed in the Dicktoo × Kompolti Korai (facultative × winter) barley population (Karsai et al., 2005b). The *Vrn-H2* gene is completely absent from the facultative parent, while it is present in the winter parent. In experiments carried out in a controlled environment chamber this gene was the main determiner of heading date under long days, irrespective of the vernalisation treatment. The vernalisation requirement could be completely linked with this gene, but in genotypes where the gene was missing, daylength was also found to have influence on heading. Both Dicktoo and Kompolti Korai carried the functional, winter allele of *Vrn-H1*, the only slight difference between the two varieties being observed in the promoter region of the gene. This difference allowed it to be mapped in this population and proved that this, or another gene closely linked to it, had an influence on the heading date, depending on the allele type of gene *Vrn-H2* (Karsai et al., 2005b).

The aim of the present experiments was to investigate the effect of major plant development genes on field tillering and to determine what other chromosome regions were involved in the regulation of tillering.

Materials and methods

Plant material

The mapping population consisted of 98 DH lines developed using the anther culture method from a Dicktoo × Kompolti Korai (DK) cross.

Phenotypic characterisation

Autumn and spring sowings were employed for the field examination of the barley population and the two parental varieties. The location of the experimental field was 47°21' N, 18°49' E, at an altitude of 150 m. Each genotype was planted as a single head-row, together with head-rows of each parent, assigned evenly over the field plan. The autumn-sown experiment was planted on 19 October 2003 and the spring-sown experiment on 19 March 2004. The mean monthly temperatures were as follows: –2.3°C in January, 1.5°C in February, 4.4°C in March and

11.2°C in April. The rainfall quantity over the vegetation period (15 October–15 July) was 474 mm, 158 mm of which fell between 15 March and 31 May. The daily photoperiod was 12 h, increasing by the date of seedling emergence in the spring-sown experiment. The plant number was reduced to 15–20 per m after overwintering in the autumn-sown experiment and after emergence in the spring-sown variant, to facilitate observations on individual plants. Plants sown in autumn were scored for tillering on three occasions: prior to the start of winter in early December (TIL1201), in spring in early April (TIL0401) and at heading (TIL49autumn). In the spring-sown crop tillering was scored by counting the number of tillers on four occasions from the appearance of the first tillers until mid-May (TIL0423, TIL0429, TIL0506, TIL0513) and at heading (TIL49spring). When scoring for heading date, the number of reproductive tillers (RTautumn and RTspring) was also recorded.

Genotypic characterisation

Several types of markers were used: the RFLP probes originated from the NABGM programme, while the STS primers developed from various RFLP probes were put at our disposal by Dr T. Blake (Montana State University, Bozeman). The RAPD primers were obtained from the OPERON Company. Information on the SSR primers is available at the following internet address: www.genetics.org. The *Vrn-H1* and *Vrn-H2* genes were mapped using specific polymorphisms located within the genes (Karsai et al., 2005b). The marker map was prepared using the MAPMAKER 3.0 program, while the programs MAPMAKER/QTL and WINQTL were used for QTL analysis. The effect of individual loci was analysed through marker regressions.

Results and discussion

Autumn sowing

Due to the cold weather in autumn, the plant stand started tillering late, at the end of November (Table 1). Although the plants only had a mean tiller number of 1.2 before the winter frost set in, overwintering averaged 85%, thanks to the mild winter. In spring the plants tillered rapidly, with a ten times increase in the number of tillers by mid-April. This intensive tillering continued until heading, and new tillers continued to form at a slower rate even after heading. The majority of tillers formed prior to heading were productive.

Among the two genes responsible for vernalisation requirement, *Vrn-H1* (5H) had no effect on tillering (Table 1), though this locus explained 21.5% of the phenotypic variance in heading date (LOD value 4.05). The minor role played by the *Vrn-H2* (4H) locus in the control of tillering only became significant at a later stage of plant development, at heading, despite the fact that this locus did not have a significant influence on the heading date after autumn sowing. By heading, lines carrying the *Vrn-H2* gene (Kompolti allele) had an average of 2.3 more tillers than lines lacking this gene (Dicktoo allele).

QTL analysis showed that in addition to the *Vrn* loci, one other chromosome region was involved in the regulation of tillering: the *HvM62 – OPS192* marker interval on the 3H chromosome (Fig. 1), which had the greatest effect at the beginning of tillering in early winter, when the allele composition of this region explained 17.3% of the phenotypic variance. This ratio dropped to 10.5% for tillering in early April. On average, lines carrying the Dicktoo marker allele had a higher tiller number at both scoring dates. By heading the 3HL region no longer had a significant effect.

Table 1

Extent of tillering in a Dicktoo × Kompolti Korai barley population after autumn and spring field sowing, and the effect of loci for vernalisation requirement on tillering

Traits	Population		*Vrn-H2* gene effect$^{(1)}$		*Vrn-H1* gene effect$^{(1)}$	
	Mean	interval	R^2 (%)	Weight	R^2 (%)	Weight
Autumn sowing 2003/04						
TIL1201 (No.)	1.2	0.6–2.1	0.2		1.8	
TIL0401 (No.)	12.5	7.8–19.3	1.6		0.4	
TIL49 (No.)	21.9	14.5–30.7	12.1***	2.3 K	0.3	
RT (No.)	23.5	15.2–32.6	2.7		0.8	
Heading$^{(2)}$	128.0	122.0–133.0	0.0		20.8***	2.4 D
Spring sowing 2004						
TIL0423 (No.)	0.7	0.0–2.4	3.5		1.2	
TIL0429 (No.)	2.5	0.7–6.7	4.7		0.3	
TIL0506 (No.)	6.2	2.5–14.3	9.6**	1.6 K	4.1	
TIL0513 (No.)	9.6	3.5–22.6	20.2***	3.2 K	5.2	
TIL49 (No.)	16.1	4.6–35.0	47.8***	8.6 K	9.9*	4.0 D
RT (No.)	11.2	3.2–22.7	26.5***	4.0 D	0.2	
Heading$^{(3)}$	80.8	67.0–103.0	73.3***	17.7 K	7.1	

[1] R^2: Determination coefficient, indicative of the proportion of phenotypic variance. *, **, ***: Gene effects significant at the P=5.0, 1.0 and 0.1% levels, respectively. Weight: effect of the parental allele with the higher value in enhancing the trait. D – Dicktoo, K – Kompolti Korai.
[2] No. of days from 1 January; [3] No. of days from sowing (19 March)

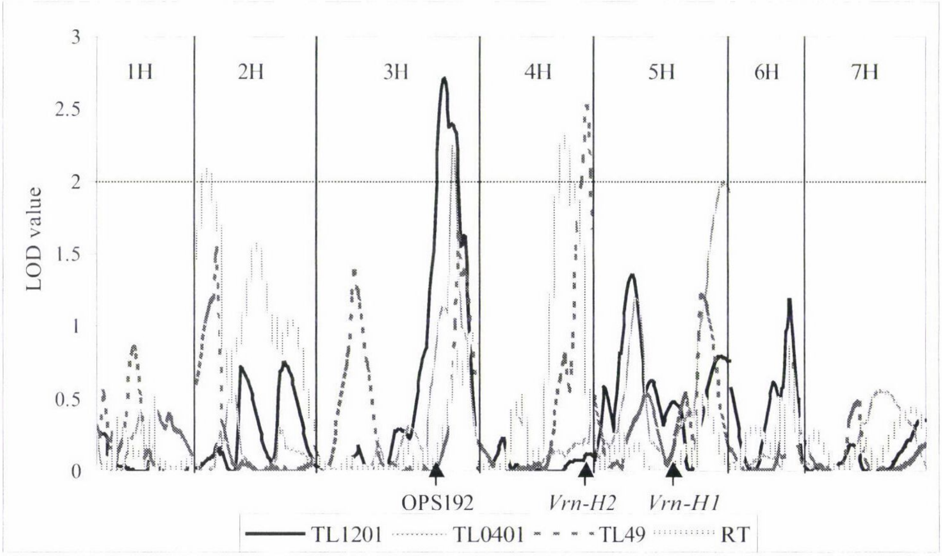

Fig. 1. QTL analysis of chromosome regions influencing the extent of tillering in a Dicktoo × Kompolti Korai population sown in autumn

Spring sowing

In the spring sowing treatment, tillering began 2–3 weeks after emergence (Table 1), after which the rate of tillering was linear for four weeks, with a further increase until heading. A comparison of the two sowing dates indicated that on average tillering vigour was somewhat poorer after spring sowing, but there were far greater differences between the lines than in the autumn sowing. There was no significant correlation between the heading date and either the total number of tillers or the number of productive tillers at any scoring date after autumn sowing. By contrast, in spring sowing the correlation between heading date and the extent of tillering became increasingly close as the plant developed, with late-heading lines having a larger number of tillers (r=0.06 for TIL0423, r=0.11 for TIL0429, r=0.20* for TIL0506, r=0.33*** for TIL0513 and r=0.58*** for TIL49spring). This depended fundamentally on the allele composition of the *Vrn-H2* gene. If there was no cold effect the vernalisation requirements of lines carrying gene *Vrn-H2* was not saturated, so they remained in the vegetative phase, resulting in a continual increase in tillering. The pleiotropic effect of the *Vrn-H2* gene on tillering became significant when the tillers were counted in the third week (Table 1). Lines carrying the *Vrn-H2* gene formed more tillers on average over the course of a week, so the difference between the two groups in the number of tillers gradually increased until heading. The *Vrn-H2* lines headed significantly later; in the DK population this difference was 17.2 days. The protracted heading of the *Vrn-H2* lines coincided with the fact that a substantial proportion of the tillers remained in the vegetative phase. Consequently, the average number of productive tillers was significantly lower than the total number of tillers at heading, while the absence of the gene (Dicktoo allele) resulted in a significantly higher number of productive tillers. As in autumn sowings, the *Vrn-H1* (5H) locus had no great influence on tillering in spring sowings. A significant effect could be detected using marker regression, but not by QTL analysis; this significant level was reached at heading, explaining 9.9% of the phenotypic variance, with a higher number of productive tillers for plants with the Dicktoo allele.

In addition to the *Vrn-H2* locus, two other QTL regions were also found to play a significant role in the control of tillering (Fig. 2), but these had no influence on the heading date. One was located on the short arm of chromosome 1H (at marker MWG938) and the other on the long arm of chromosome 3H (in the HvM62 – OPS192 marker interval). The latter locus was also involved in the regulation of tillering in autumn sowing. In spring sowing there was an epistatic interaction between the *Vrn-H2* locus and both of the tillering QTL regions (Table 2). The 3HL locus only participated in the control of tillering in lines from which *Vrn-H2* was absent. In this case lines with the Kompolti allele at the OPS192 marker had smaller tillering vigour right from the beginning (Fig. 3). The 1HS region had a weaker effect than the 3HL region and only influenced tillering in lines carrying the *Vrn-H2* gene, resulting in a significantly higher number of tillers in lines with the Kompolti allele at marker MWG938. Both QTL regions had a greater effect at the beginning of tillering, gradually losing influence as the plants developed (Fig. 4). This decline coincided with the strengthening of the effect of gene *Vrn-H2* on tillering.

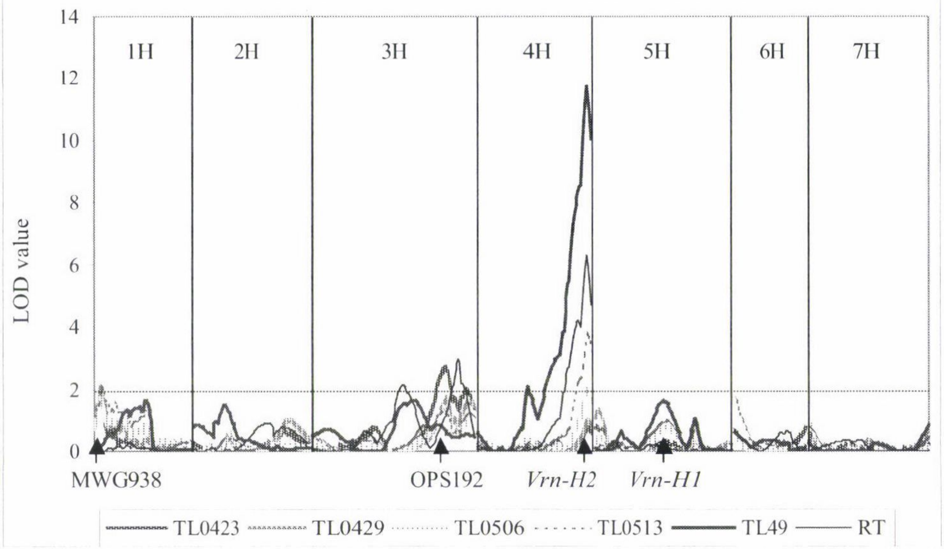

Fig. 2. QTL analysis of chromosome regions influencing the extent of tillering in a Dicktoo × Kompolti Korai population sown in spring

Table 2

Interaction between the *Vrn-H2* locus and the allelic composition of the tillering QTL regions in the Dicktoo × Kompolti Korai barley population, based on marker regression

Traits	Effect of the 3HL (HvM62-OPS192) QTL locus				Effect of the 1HS (MWG938) QTL locus			
	Vrn-H2–		*Vrn-H2+*		*Vrn-H2–*		*Vrn-H2+*	
	R^2 (%)	Weight	R^2 (%)	Weight	R^2 (%)	Weight	R^2 (%)	Weight
TIL1201	10.3*	0.2 D	9.1*	0.2 D	2.1		0.0	
TIL0401	10.2*	1.4 D	9.1*	1.3 D	1.2		2.9	
TIL49autumn	0.2		6.9		0.9		7.7	
RTautumn	0.4		8.6		5.6		2.3	
TIL0423	30.2***	0.6 D	2.6		1.6		21.5**	0.6 K
TIL0429	20.9**	0.8 D	0.8		2.1		17.1**	0.9 K
TIL0506	17.7**	1.8 D	0.5		1.1		13.0*	1.9 K
TIL0513	13.5*	2.0 D	0.1		2.1		11.9*	2.7 K
TIL49spring	0.3		3.8		0.5		0.9	
RTspring	1.1		15.8**	3.0 K	2.0		4.5	

*, **, ***: Gene effects significant at the P=5.0, 1.0 and 0.1% levels, respectively.

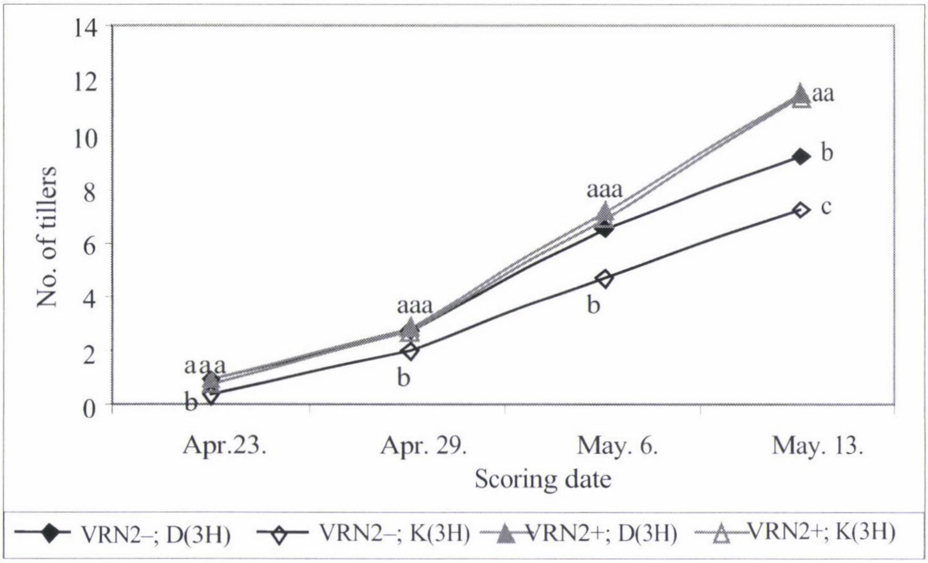

Fig. 3. Effect of the interaction between the Vrn-H2 locus and the tillering QTL region identified on the long arm of chromosome 3H on the rate of tillering in spring-sown barley, based on the mean tillering of the four marker groups in the Dicktoo × Kompolti Korai population (means designated by the same letter did not differ significantly at the P=5% level)

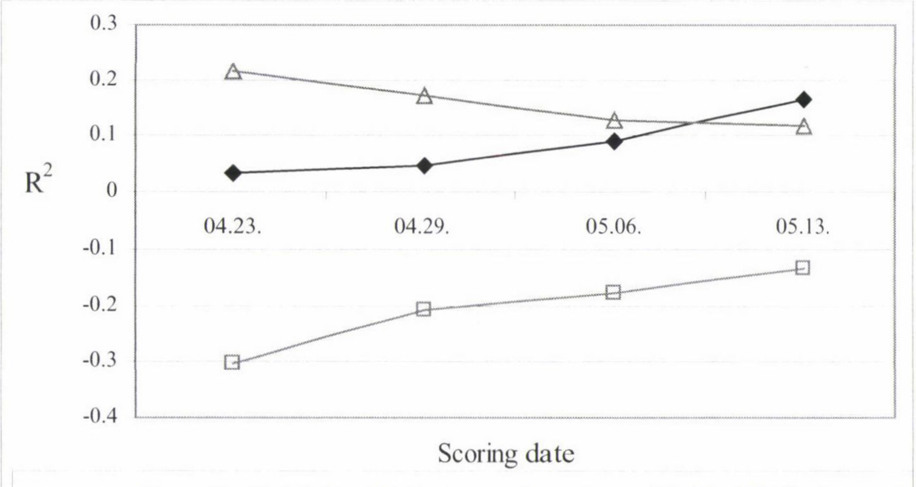

Fig. 4. Role of various chromosome regions in the determination of the tillering rate in a Dicktoo × Kompolti Korai population sown in the field in spring (R^2 = determination coefficient, the sign of which indicates the parental allele resulting in the higher tillering rate; positive numbers: Kompolti Korai; negative numbers: Dicktoo)

It can be concluded from the results that major ontogenic genes also have a significant effect on the tillering rate. This regulatory function is achieved partly directly, through their effect on the transition from the vegetative to the generative phase, and partly indirectly, through the epistatic regulation of other chromosome regions influencing tillering. Among the major ontogenic genes, the *Vrn-H2* locus had the greatest effect on tillering in the facultative × winter barley population investigated, and in later stages of ontogeny, as heading was approached, this effect became increasingly important, irrespective of the sowing date. The repressor protein produced by the *Vrn-H2* gene not only regulated the functioning of the *Vrn-H1* gene, but also had an epistatic interaction with the two tillering QTL regions. Neither of the chromosome regions responsible for tillering, identified in the present work, had a significant effect on the heading date, in either autumn or spring sowing. Nevertheless, the possibility that these regions play some role in the regulation of ontogeny cannot be completely ruled out. For one thing, they exerted their greatest effect during the initial stages of ontogeny, but were later masked by the increasing activity of the *Vrn-H2* gene. In addition, genetic analyses have revealed numerous minor regions involved in the control of heading date in cereals, for which neither the exact mechanism nor their role in the control of flowering have yet been determined. These include the earliness genes (*eps*) identified on almost all the chromosomes of barley and the *ea* genes that influence the heading date (Laurie et al., 1995; Murai et al., 1997; Buck-Sorlin and Börner, 2001). For instance, earliness genes have been described at loci on the long arm of chromosome 3H (ea_{sp}, Gallagher et al., 1991; *eps3L*, Laurie et al., 1995), the physical location of which with respect to each other is uncertain, due to the lack of a joint marker map, but which may coincide with the QTL loci for tillering identified in the present work on 3HL. However, the site of the tillering QTL found on the distal end of 1HS has not yet been implicated in ontogeny. Although the present results indicate an epistatic relationship between these two QTL regions and the *Vrn-H2* gene, special basic genetic stocks will be required to give an accurate picture of the genetic control of tillering.

References

Buck-Sorlin, G. H., Börner, A. (2001): Pleiotropic effects of the ea7 photoperiod response gene on the morphology and agronomic traits in barley. *Plant Breeding*, **120**, 489–495.

Danyluk, J., Kane, N. A., Breton, G., Limin, A. E., Fowler, D. B., Sarhan F. (2003): TaVRT-1, a putative transcription factor associated with the vegetative to reproductive transition in cereals. *Plant Physiol.*, **132**, 1849–1860.

Dubcovsky, J., Chen, C., Khan, I. A., Yan, L. (2005): Molecular characterization of the allelic variation at the *VRN-H2* vernalization locus in barley. *Mol. Breed.*, **15**, 395–407.

Gallagher, L. W., Soliman, K. M., Vivar, H. (1991): Interaction among loci conferring photoperiod insensitivity for heading time in spring barley. *Crop Sci.*, **31**, 256–261.

Karsai, I., Mészáros, K., Szűcs, P., Hayes, P. M., Láng, L., Bedő, Z. (1999): Effects of loci determining photoperiod sensitivity (*Ppd-H1*) and vernalization response (*Sh2*) on agronomic traits in the Dicktoo × Morex barley mapping population. *Plant Breeding*, **118,** 399–403.

Karsai, I., Mészáros, K., Láng, L., Bedő, Z. (2005a): Changes in agronomic traits affected by photoperiod and vernalization in a group of wild barley accessions (*Hordeum vulgare* ssp. *spontaneum*) and barley cultivars (*Hordeum vulgare* L.). *Acta Agron. Hung.*, **53**, 89–98.

Karsai, I., Szűcs, P., Mészáros, K., Filichkina, T., Hayes, P. M., Skinner, J. S., Láng, L., Bedő, Z. (2005b): The *Vrn-H2* locus is a major determinant of flowering time in a facultative × winter growth habit barley (*Hordeum vulgare* L.) mapping population. *Theor. Appl. Genet.*, **110**, 1458–466.

Kato, K., Miura, H., Sawada, S. (2000): Mapping QTLs controlling grain yield and its components on chromosome 5A of wheat. *Theor. Appl. Genet.*, **101,** 1114–1121.

Laurie, D. A., Pratchett, N., Bezant, J. H. Snape, J. W. (1994): Genetic analysis of a photoperiod response gene on the short arm of chromosome 2(2H) of *Hordeum vulgare* (barley). *Heredity*, **72**, 619–627.

Laurie, D. A., Pratchett, N., Bezant, J. H., Snape, J. W. (1995): RFLP mapping of five major genes and eight quantitative trait loci controlling flowering time in a winter × spring barley (*Hordeum vulgare* L.) cross. *Genome*, **38**, 575–585.

Murai, K., Koba, T., Shimada, T. (1997): Effects of barley chromosome on heading characters in wheat-barley chromosome addition lines. *Euphytica*, **96**, 281–287.

Pan, A., Hayes, P. M., Chen, F., Chen, T. H. H., Blake, T., Wright, S., Karsai, I., Bedő, Z. (1994): Genetic analysis of the components of winterhardiness in barley (*Hordeum vulgare* L.). *Theor. Appl. Genet.*, **89**, 900–910.

Snape, J. W., Law, C. N., Parker, B. B., Worland, A. J. (1985): Genetical analysis of chromosome 5A of wheat and its influence on important agronomic characters. *Theor. Appl. Genet.*, **71**, 518–526.

von Zitzewitz, J., Szűcs, P., Dubcovsky, J., Yan, L., Pecchioni, N., Francia, E., Casas, A., Chen, T. H. H., Hayes, P. M., Skinner, J. S. (2005): Molecular and structural characterization of barley vernalization genes. *Plant Mol. Biol.*, **59**, 449–467.

Worland, A. J. (1996): The influence of flowering time genes on environmental adaptabilitiy in European wheats. *Euphytica*, **89**, 49–57.

Yan, L., Loukoianov, A., Tranquilli, G., Helguera, M., Fahima, T., Dubcovsky, J. (2003): Positional cloning of the wheat vernalization gene VRN1. *PNAS*, **100**, 6263–6268.

Yan, L., Loukoianov, A., Blechl, A., Tranquilli, G., Ramakrishna, W., SanMiguel, P., Bennetzen, J. L., Echenique, V., Dubcovsky, J. (2004): The wheat VRN2 gene is a flowering repressor down-regulated by vernalization. *Science*, **303**, 1640–1644.

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DIFFERENTIAL RESPONSE OF TWO *Vicia faba* CULTIVARS TO DROUGHT: GROWTH, PIGMENTS, LIPID PEROXIDATION, ORGANIC SOLUTES, CATALASE AND PEROXIDASE ACTIVITY

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A study on the germination of five *Vicia faba* cultivars exposed to polyethylene glycol-induced water stress indicated that cv Giza 40 showed the highest germination capacity and cv Giza 667 the lowest. The effect of low soil water content was studied on the plant growth, photosynthetic pigment content, organic solutes, relative water content (RWC), lipid peroxidation, membrane stability index (MSI), and the catalase (CAT) and peroxidase (POX) activity in the leaves of 21-day-old *Vicia faba* cv Giza 40 and cv Giza 667 plants. With respect to dry weight (DW), drought caused a greater decrease in cv Giza 667 than in cv Giza 40, indicating that cv Giza 40 was more tolerant of low soil water content. Drought decreased the Chl a, Chl b and carotenoid contents and the Chl a/b and carotenoid/Chl a+b ratios in the leaves of cv Giza 667, while in cv Giza 40 a significant increase in these pigment parameters was observed under drought stress. Drought caused a decrease in RWC and MSI and an increase in the lipid peroxidation level and in the catalase (CAT) and peroxidase (POX) activity in both the cultivars, but the decline in RWC and MSI and the increase in lipid peroxidation level in response to drought stress were greater in cv Giza 667 than in cv Giza 40. The CAT and POX activities were higher in Giza 40 than in Giza 667 under both control and drought conditions. Drought induced the accumulation of soluble sugars, soluble proteins, free amino acids and proline in both cultivars. However, this accumulation was lower in cv Giza 667 than in the more tolerant cv Giza 40. These results indicate that cv Giza 40 showed better protection against drought-induced oxidative stress through higher CAT and POX activities and osmolyte concentrations than cv Giza 667.

Keywords: catalase, drought, lipid peroxidation, peroxidase, *Vicia faba*

Introduction

Drought is an important environmental factor, which induces significant alterations in plant physiology and biochemistry. Some plants exhibit a number of physiological adaptations that allow them to tolerate water stress conditions. The degree of adaptation to the decrease in water potential caused by drought may vary considerably between species (Savé et al., 1995) and also within a species (Parker and Pallardy, 1985). The most common symptom of water stress injury is the decrease in seed germination (Schmidhalter and Oertli, 1991) and the inhibition of growth, which is reflected in a reduction in the dry matter yield (Schmidhalter and Oertli, 1991; El-Tayeb and Hassanein, 2000; Le Thiec and Manninen, 2003). The inhibition of plant growth under water stress conditions is associated with altered water relations (Torrecillas et al., 1995; Dichio et al., 2003). Drought was found to decrease the relative water content of plant leaves (Sánchez-Blanco et al., 2002). Total chlorophyll, carotenoid content and the Chl a/b ratio were found to decline under water deficit conditions. The rate of decline in a drought-sensitive cultivar was much faster than in a more drought-resistant cultivar (Shaddad and El-Tayeb, 1990). However, Sestak and Vaclavick (1965) pointed out that the chlorophyll content may increase under water stress conditions. It was found that drought influenced the carbohydrate and nitrogen metabolism. Some drought-stressed plants accumulate carbohydrates in soluble form, which is related to the osmoregulation metabolism (Zrenner and Stitt, 1991). Drought was found to induce the accumulation of soluble proteins and free amino acids, including proline, in various plants (Vyas et al., 1985; Girousse et al., 1996; Ain-Lhout et al., 2001). Proline accumulation varied with the degree of plant resistance to drought (Levy, 1983). Therefore, proline could be used for the evaluation of the tolerance or sensitivity of plants to stress (Patel and Vora, 1984). In addition, Claussen (2005) reported that proline was a reliable indicator of the environmental stress suffered by plants and established stress thresholds for the fruit yield and product quality of tomato plants.

Drought induces the generation of reactive oxygen species, causing lipid peroxidation, and consequently membrane injury, protein degradation, enzyme inactivation and the disruption of DNA strands (Imlay and Linn, 1988; Becana et al., 1998). The rapid removal of toxic oxygen radicals is of prime importance in any defence mechanism. Plants protect cells and subcellular systems from the cytotoxic effects of these active oxygen radicals through both non-enzymatic and enzymatic antioxidant systems such as carotenoids, ascorbic acid, α-tocopherol, POX and CAT (Munné-Bosch and Alegre, 2000; Sairam and Srivastava, 2001; Fu and Huang, 2001). Many reports underline the intimate relationship between antioxidant enzyme activities and increased tolerance to environmental stress (Sgherri et al., 2000; Sairam et al., 2002; Bor et al., 2003; Reddy et al., 2004; Türkan et al., 2005).

Therefore, the present work was conducted to detect which cultivar of *Vicia faba* could germinate and sustain growth under water stress conditions, and to detect the physiological mechanisms underlying the differential tolerance of two *Vicia faba* cultivars to drought. The changes in growth, photosynthetic pigments, relative water content (RWC), membrane stability index (MSI), lipid peroxidation, antioxidant POX and CAT activity and the content of soluble sugars, soluble proteins, total free amino acids and free proline in the relatively drought-tolerant *V. faba* cv Giza 40 and the relatively drought-sensitive cv Giza 667 were studied.

Materials and methods

The seeds of five *Vicia faba* cultivars (Giza 40, Giza 67, Giza 102, Giza 103 and Giza 667) were obtained from the Agricultural Research Center and the Faculty of Agriculture, Assiut University, Egypt. A preliminary experiment was carried out to study the effect of water stress on the seed germination of the different cultivars to detect the most tolerant and the most sensitive to water stress. The water stress levels [0.0 (control), –0.05, –0.1, –0.2, –0.4 and –0.6 MPa] were achieved using polyethylene glycol (PEG, 6000 MW) in 1/10 Hoagland solution (Hoagland and Arnon, 1950). The seeds in the control group were germinated in 1/10 Hoagland solution. The seeds of all genotypes were sterilized in 5% Clorox solution (sodium hypochlorite) for 5 min followed by a 5 min dip in 70% ethanol. The seeds were washed three times with sterilized distilled water and left to germinate in Petri dishes in the dark at 25°C (20 seeds per dish). Three replicates were prepared for each treatment.

Seeds of *Vicia faba* L. cv Giza 40 (relatively drought-tolerant) and cv Giza 667 (relatively drought-sensitive) were then sown in plastic pots containing 2 kg air-dried soil (sand/clay 1:1 v/v) per pot. The pots were watered with ½ strength Hoagland solution and then adjusted to the desired soil water content [90% (control) and 40% (drought) of maximum field capacity]. Twenty seeds were used per pot. Three pots were assigned to each treatment. The plants were irrigated every other day with distilled water to the desired moisture level. The plants were left to grow under controlled conditions (light/dark regime of 12/12 h at 22/16°C, light intensity 3040 lux) for three weeks. At the end of the experimental period the plants were harvested. Half of the samples were rapidly dried in an oven at 80ºC to constant weight and then ground to powder, which was used for the determination of dry weight and further analysis. Another part was used immediately for pigment extraction, and the estimation of the relative water content (RWC) and membrane stability index (MSI) of the leaves. The remaining samples were frozen in liquid nitrogen and stored at –20°C for biochemical analysis.

The photosynthetic pigments (chlorophyll a, chlorophyll b, carotenoids) were determined according to Metzner et al. (1965). Leaf material (0.1 g each) of young, fully expanded leaves of three plants from each replicate were used for pigment extraction. The pigment extract was measured against a blank of pure 85% acetone at wavelengths of 452.5, 644 and 663 nm.

Leaf relative water content (RWC) was estimated according to the method of Whetherley (1950). The leaf material was weighed (0.5 g) to determine fresh weight and placed in double distilled water for 4 h, after which the turgid weight was recorded. Finally, the samples were dried in an oven at 65°C for 48 h and the dry weights were recorded. RWC was calculated as:

$$\text{RWC} = [(\text{Fresh weight} - \text{Dry weight})/(\text{Turgid weight} - \text{Dry weight})] \times 100.$$

The membrane stability index (MSI) was determined according to Sairam et al. (2002). Leaf samples (0.1 g each) were cut into discs of uniform size and placed in 10 ml of double distilled water in two sets. One set was kept at 40°C for 30 min and its conductivity recorded (C_1) using a conductivity meter (HANNA HI 991300). The second set was kept in a boiling water bath (100°C) for 15 min and its conductivity also recorded (C_2). The membrane stability index (MSI) was calculated as:

$$\text{MSI} = [1-(C_1/C_2)] \times 100.$$

The level of lipid peroxidation in leaf tissue was measured by the determination of MDA, a breakdown product of lipid peroxidation. The MDA content was determined with the thiobarbituric acid reaction. Briefly, 0.25 g of frozen sample was homogenized in 5 ml 0.1% TCA. The homogenate was centrifuged at 10,000 g for 5 min, after which 4 ml of 20% TCA containing 0.5% TBA was added to a 1 ml aliquot of the supernatant. The mixture was heated at 95°C for 15 min and then cooled immediately. The developed colour compound was extracted with 2 ml n-butanol and the absorbance was measured at 532 nm. The value for the non-specific absorption at 600 nm was subtracted. The level of lipid peroxidation was expressed as nmole of MDA formed using an extinction coefficient of 155 mmol L^{-1} cm^{-1} (Zaho et al., 1994).

For the assays of CAT and POX enzymes, 200 mg leaves was homogenized in an ice-cooled mortar, ground in 1 ml of 100 mM K phosphate buffer (pH 7.4) and centrifuged at 10,000 g for 10 min under cooling; the supernatant was used for enzyme assay. The activities of CAT and POX were determined according to Chance and Maehly (1955). CAT activity was determined by measuring the decomposition of H_2O_2, and the decline in absorbance at 240 nm was followed for 3 min. The 3-ml reaction mixture contained 50 mM phosphate buffer (pH 7.0), 15 mM H_2O_2 and 0.1 ml of enzyme extract, which started the reaction. The activity of POX, assayed by measuring the oxidation of guaiacol and the increase in absorbance at 470 nm, was recorded for 3 min. The reaction mixture contained 50 µl of 20 mM guaiacol, 2.8 ml of 10 mM phosphate buffer (pH 7.0) and 0.1 ml enzyme extract. The reaction was started with 20 µl of 40 mM H_2O_2. The activity was defined as OD/min/mg FW.

The water-soluble sugars were quantified by the anthrone sulphuric acid method (Fales, 1951). Powdered samples (100 mg each) of dried leaves were heated in a water bath at 100°C for 2 h in 10 ml distilled H_2O. The solution was cooled, transferred to a 50 ml measuring flask after filtration through a centered glass funnel, and made up to a known volume with distilled water. Then 4.5 ml of anthrone reagent was added to 0.5 ml of the prepared solution in a clean dried test tube, and the soluble sugar content was calculated as mg g^{-1} dry weight of the plant leaves. To estimate soluble proteins, powdered leaf samples (100 mg each) were boiled in 10 ml distilled water for two hours. After cooling, the water extract was centrifuged, the supernatant was decanted and made up to a known volume using distilled water, and the soluble proteins were determined according to Lowry et al. (1951). Free amino acids were extracted from leaf tissues and determined according to the method of Moore and Stein (1948), while free proline was determined according to Bates et al. (1973). A spectrophotometer of the Genway 6405 UV/Visible type was used for the determinations.

The data were statistically analysed by one-way analysis of variance using the PCSTAT program. The least significant difference (LSD) method was used to test the difference between the treatments.

Results

The results obtained for the germination of different *Vicia faba* cultivars treated with various water stress levels are given in Table 1. The final germination percentage was significantly unaffected up to –0.1 MPa in three cultivars (Giza 102, 103 and 677) and up to –0.2 MPa in two cultivars (Giza 40 and 67). Higher stress levels significantly decreased the germination percentages for the five cultivars as compared with those under control conditions. At the highest stress level used (–0.6 MPa) the highest germination percentage (36.67%) was recorded for Giza 40 and the lowest value (11.67%) for Giza 667. This may indicate that Giza 40 was the most drought-resistant cultivar, while Giza 667 was the most drought-sensitive.

Table 1

Final germination percentage of five *Vicia faba* cultivars as affected by different levels of PEG-induced water stress. Each value is the mean of three replicates

Osmotic potential [MPa]	*Vicia faba* cultivars				
	Giza 40	Giza 67	Giza 102	Giza 103	Giza 667
–0.00 (Control)	100^a	100^a	100^a	100^a	100^a
–0.05	100^a	100^a	100^a	100^a	100^a
–0.10	96.67^a	96.67^a	91.67^b	93.33^b	93.33^b
–0.20	80^b	78.33^b	76.67^b	80^b	73.67^b
–0.40	61.67^b	50^b	46.67^b	48.33^b	40^b
–0.60	36.67^b	21.67^b	18.33^b	23.33^b	11.67^b
$\text{LSD}_{5\%}$	5.05	4.68	3.82	3.31	4.27
$\text{LSD}_{1\%}$	7.01	6.49	5.30	4.59	5.93

[a] Value not significant, [b] significantly different from the corresponding control at P=0.05

Seeds of Giza 40 and Giza 667 were selected for further studies and sown in soils containing 90% (control) or 40% of field water capacity. The effect of drought on the leaves of the two cultivars was monitored. The seedling dry mass of both cultivars decreased significantly under drought conditions. However, the dry mass was higher for the more drought-tolerant cultivar (Giza 40) (Table 2). The results for chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoids (Carot.) for both cultivars under control and drought conditions are given in Table 2. The data showed that drought reduced the content of all pigment fractions in cv Giza 667. On the other hand, Chl a, Chl b, carotenoids and total pigment content increased considerably in the leaves of stressed cv Giza 40 compared with those of the control. In addition, the leaves of the more drought tolerant cultivar (Giza 40) showed higher values of Chl a+b and the Chl a/b and Carot/Chl a+b ratios in the stressed than in the control plants (Table 2). In contrast to the more drought-tolerant cultivar, under stress conditions a significant decline in all the pigment criteria was found for Giza 667.

To understand how the water status and membrane integrity of the two cultivars were affected by drought the relative water content (RWC) and membrane stability index (MSI) of the leaves of both cultivars were monitored at 90% and 40% field water capacity. The RWC in the leaves of both cultivars decreased significantly under stress conditions (Table 2). Giza 40 maintained higher RWC (69.865) than Giza 677 (62.769) under stress conditions. The MSI in the leaves of Giza 40 decreased from 88.815 to 75.045 in response to water stress, while it decreased from 87.33 to 58.083 in the case of Giza 667 (Fig. 1). The lipid peroxidation level in the leaves of the two broad bean cultivars was measured as the content of MDA and is represented in Figure 2. Under drought conditions, the MDA content increased significantly in the leaves of both cultivars. Comparatively, the MDA content was lower in the more resistant cultivar (Giza 40) than in the more sensitive one (Giza 667).

Table 2

Effect of soil moisture content on plant dry weight (g plant^{-1}), leaf relative water content (RWC) and contents of Chl a, Chl b and carotenoids (mg g^{-1} FW) of 21-day-old *Vicia faba* cv Giza 40 and cv Giza 667 plants. Each value is the mean of three replicates (3 plants each)

Line	Field capacity	Dry wt.	RWC%	Chl a	Chl b	Carot.	Total	Chl (a+b)	Chl a/b	Carot./ Chl(a+b)
Giza 40	90%	1.156[a]	87.157[a]	0.243[a]	0.397[a]	0.037[a]	0.676[a]	0.639[a]	0.610[a]	0.057[a]
	40%	0.739[b]	69.865[b]	0.322[b]	0.437[b]	0.088[b]	0.848[b]	0.760[b]	0.740[b]	0.117[b]
	$\text{LSD}_{5\%}$	0.106	5.901	0.021	0.011	0.0074	0.0173	0.012	0.0179	0.01
	$\text{LSD}_{1\%}$	0.176	8.940	0.034	0.019	0.0122	0.0286	0.021	0.0297	0.017
Giza 667	90%	0.943[a]	88.312[a]	0.322[a]	0.427[a]	0.095[a]	0.844[a]	0.749[a]	0.752[a]	0.126[a]
	40%	0.473[b]	62.769[b]	0.277[b]	0.403[b]	0.064[b]	0.744[b]	0.680[a]	0.686[b]	0.094[b]
	$\text{LSD}_{5\%}$	0.058	5.691	0.0091	0.014	0.0103	0.036	0.072	0.0136	0.01
	$\text{LSD}_{1\%}$	0.096	8.622	0.015	0.024	0.017	0.059	0.119	0.0227	0.017

[a] Value not significant, [b] significantly different from the corresponding control at P=0.05.

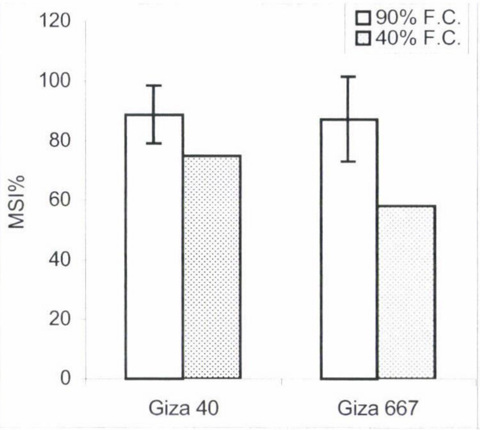

Fig. 1. Effect of soil moisture content on the membrane stability index (MSI%) in the leaves of 21-day-old *Vicia faba* cv Giza 40 and cv Giza 667 plants. Data are the means of three replicates (3 plants each). Vertical lines indicate the LSD at P=0.05 for the corresponding control

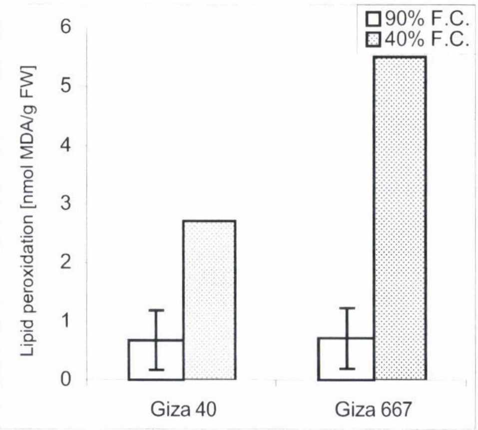

Fig. 2. Effect of soil moisture content on the lipid peroxidation level (nmol MDA/g FW) in the leaves of 21-day-old *Vicia faba* cv Giza 40 and cv Giza 667 plants. Data are the means of three replicates. Vertical lines indicate the LSD at P=0.05 for the corresponding control

To test the effect of drought on the enzymatic antioxidant system the activity of the representative enzymes catalase (CAT) and peroxidase (POX) were monitored and are given in Figures 3 and 4. The data showed that the POX activity increased slightly in cv Giza 40, but significantly in the case of Giza 667 under drought conditions (Fig. 4). The CAT activity was significantly induced in both cultivars under drought stress. However, cv Giza 40 had higher POX and CAT activity under control conditions (Figs. 3 and 4).

The contents of soluble sugars, soluble proteins and free amino acids, including proline, in the leaves of the two cultivars at the 90% and 40% field water capacity levels are given in Table 3. Soluble sugars and soluble proteins accumulated significantly in the leaves of both lines in response to drought stress. However, the more resistant cv Giza 40 showed greater accumulation of soluble sugars and proteins than cv Giza 667. Drought induced no significant changes in the free amino acid content in the leaves of Giza 667, while in those of Giza 40 a significant increase in the free amino acid content was observed under water stress conditions (Table 3). The free proline content increased significantly when leaves of the two cultivars were subjected to drought stress. However, Giza 40 accumulated more proline than Giza 667 under both control and drought conditions.

Discussion

Through its osmotic effect drought induces various responses in natural and agricultural habitats, such as the inhibition of germination and growth and the synthesis of non-toxic compounds that increase the osmotic potential of the cell and thus allow metabolic processes to continue.

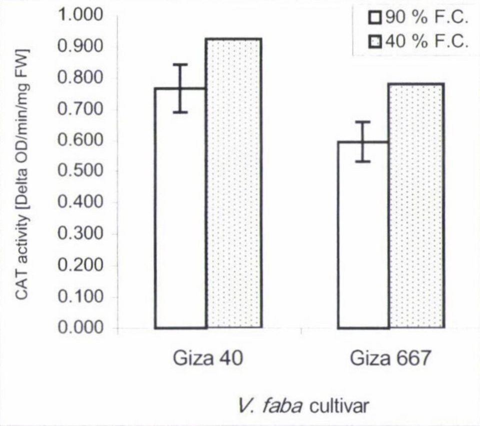

Fig. 3. Effect of soil moisture content on the catalase (CAT) activity (ΔOD/min/mg FW) in the leaves of 21-day-old *Vicia faba* cv Giza 40 and cv Giza 667 plants. Data are the means of three replicates. Vertical lines indicate the LSD at P=0.05 for the corresponding control.

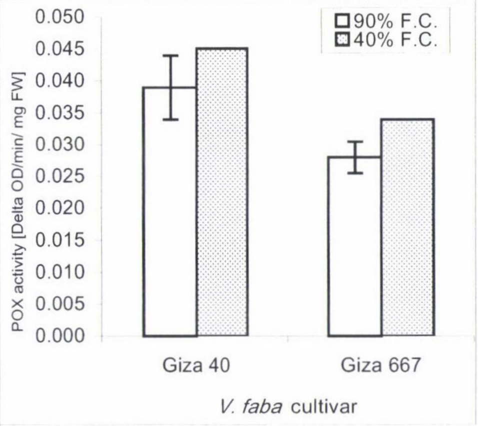

Fig. 4. Effect of soil moisture content on the peroxidase (POX) activity (ΔOD/min/mg FW) in the leaves of 21-day-old *Vicia faba* cv Giza 40 and cv Giza 667 plants. Data are the means of three replicates. Vertical lines indicate the LSD at P= 0.05 for the corresponding control

In the present study, although the germination percentage decreased progressively with an increasing level of PEG-induced water stress, Giza 40 was the most tolerant cultivar of the *Vicia faba* cultivars tested and Giza 667 the most sensitive one (Table 1). This inhibitory effect of water stress on seed germination was reported by other authors using various plants (Schmidthalter and Oertli, 1991; El-Tayeb and Hassanein, 2000; Schütz et al., 2002). In addition, Bayuelo-Jimenez et al. (2002) found that the germination capacity was higher in *Phaseolus filiformis* (more drought-resistant) than in *Phaseolus vulgare* (more drought-sensitive) under water stress.

Table 3

Effect of soil moisture content on the content of soluble sugars, soluble proteins, free amino acids and proline (mg g^{-1} DW) in the leaves of two (21-day-old) *Vicia faba* cultivars. Each value is the mean of three replicates

Cultivar	Field capacity	Soluble sugars	Soluble proteins	Total free amino acids	Proline
Giza 40	90%	16.27[a]	86.675[a]	31.065[a]	5.15[a]
	40%	32.21[b]	116.65[b]	33.015[b]	7.89[b]
	$LSD_{5\%}$	2.12	9.20	0.578	0.453
	$LSD_{1\%}$	3.52	15.26	0.958	0.751
Giza 667	90%	11.32[a]	65.965[a]	30.18[a]	4.95[a]
	40%	14.71[b]	111.73[b]	30.48[a]	6.84[b]
	$LSD_{5\%}$	7.95	7.14	NS	0.792
	$LSD_{1\%}$	13.19	11.84	NS	1.248

[a] Value not significant, [b] significantly different from the corresponding control at P=0.05.

The study on drought using soil culture showed that cv Giza 40 was also more tolerant than cv Giza 667 with regard to growth. Drought treatment (40% F.C.) caused a decrease in seedling dry mass in both the cultivars. These results are in agreement with those obtained by other authors (Shaddad and El-Tayeb, 1990; Kirnak et al., 2001). Comparatively, cv Giza 40 showed higher dry leaf mass than cv Giza 667 (Table 2). Working with two wheat cultivars Loggini et al. (1999) found that drought caused a more pronounced inhibition in growth in the more drought-sensitive cv Adamello compared with the relatively drought-tolerant cv Ofanto. In addition, Türkan et al. (2005) reported that the biomass production of *Phaseolus acutifolius* (more drought-tolerant) was superior to that of *Phaseolus vulgaris* (more drought-sensitive) under water stress. In general, drought resulted in a significant decrease in the RWC in the leaves of both cultivars. However, the more tolerant cv Giza 40 showed higher RWC than the more sensitive cv Giza 667 under drought stress (Table 2). Previous works also reported that drought-tolerant genotypes exhibited higher RWC compared to drought-sensitive genotypes in several species, including wheat (Sairam and Srivastava, 2001), moth bean (Garg et al., 2001), barley (Kocheva and Georgiev, 2003) and *Phaseolus* (Türkan et al., 2005).

It is well known that drought enhances free radical production, which induces the lipid peroxidation of biomembranes, reflecting the stress-induced damage in tissues. The MDA content is often used as an indicator of the extent of lipid peroxidation resulting from oxidative stress (Smirnoff, 1993). In the present work, the MDA content significantly increased in the leaves of the two *V. faba* cultivars in response to drought stress, but cv Giza 40 showed lower MDA accumulation than cv Giza 667 (Fig. 2). In addition, the values of the membrane stability index (MSI) decreased to a lesser extent in Giza 40 under drought stress than in Giza 667 (Fig. 1). This is in agreement with the work of Sairam and Srivastava (2001), who reported that drought-tolerant genotypes of wheat showed a lower lipid peroxidation level and higher MSI than susceptible ones. In addition, Türkan et al. (2005) found that the MDA content was lower in the leaves of drought-tolerant *Phaseolus acutifolius* Gray than in drought-sensitive *P. vulgaris* L. The lower increase in MDA and the lower decrease in MSI in drought-stressed cv Giza 40 suggests better protection from oxidative damage, apparently resulting from the more efficient antioxidative system, while the higher accumulation of MDA and lower MSI in the leaves of cv Giza 667 could be attributed to a less efficient antioxidant system. These results are in agreement with the results of Sairam et al. (2002) and Bor et al. (2003), who found a correlation between increased antioxidant enzyme activities and decreased lipid peroxidation in drought-tolerant wheat, *Triticum aestivum* cv Kharchia 65 and wild beet, *Beta maritima* under salt stress.

Drought, like other environmental stresses such as high and low temperatures and salinity, also induces oxidative stress. To be able to endure oxidative damage under unfavourable conditions, plants possess both non-enzymatic antioxidants such as carotenoid, flavonoids, α-tocopherol, ascorbic acid and glutathione, and enzymatic antioxidants such as CAT and POX (Smirnoff, 1993; Munné-Bosch and Alegre, 2000). These enzymatic and non-enzymatic antioxidants are reported to accumulate under various environmental stresses (Yu and Rengel, 1999; Acar et al., 2001), while comparatively higher activity has been reported in tolerant cultivars than in sensitive ones (Sairam et al., 2002; Reddy et al., 2004), indicating that higher antioxidant enzyme activity has a role in imparting tolerance against environmental stress. In the light of this, the higher CAT and POX activities in cv Giza 40 under drought stress indicate its relative tolerance to drought, while cv Giza 667 was inferior on this count.

Catalase (CAT) and peroxidase (POX) play an essential role in protection against H_2O_2 toxicity. Under drought, the CAT and POX activities increased significantly in the leaves of Giza 667, while only CAT increased significantly in the case of cv Giza 40 (Figs. 3 and 4). However, the level of CAT and POX activity was higher in the tolerant cv Giza 40 than in the sensitive cv Giza 667, suggesting a higher capacity to decompose H_2O_2 under drought stress. These results are in good agreement with the results of Reddy et al. (2004) and Türkan et al. (2005), who found a higher constitutive level of CAT and POX activity in drought-tolerant mulberry cvs S-13 and BC2-59 and in *Phaseolus acutifolius*, respectively.

The contents of Chl a, Chl b and Carot. pigments, total chlorophyll (a+b), and Chl a/b and Carot/Chl a+b ratios decreased in the leaves of the more drought-sensitive cv Giza 667 under drought conditions (Table 2). These results are in accordance with those obtained by other authors (Loggini et al., 1999; Barathi et al., 2001; Fu and Huang, 2001; Egert and Tevini, 2002). Photoinhibition and the photodestruction of pigments may contribute to such alterations. On the other hand, in the case of cv Giza 40 the pigment content increased in stressed plants. Sestak and Vaclavick (1965) pointed out that the chlorophyll content may increase under conditions of water deficit. In addition, García-Valenzuela et al. (2005) reported that a chlorophyllic cell line ('TADH-XO') of the highly water stress-tolerant grass *Bouteloua gracilis* developed substantially higher amounts of chlorophyll (a and b) when subjected to PEG-induced water stress. In the present work, the drought-induced increase in chlorophyll a and b in cv Giza 40 was associated with a significant increase in the carotenoid content and in the value of the Carot/Chl a+b ratio, indicating the capacity to protect the photosynthetic apparatus. Loggini et al. (1999) reported that carotenoids are involved in the protection of the photosynthetic apparatus against photoinhibitory damage by singlet oxygen ($^1O_2$), which is produced by the excited triplet state of chlorophyll.

The results also showed that in cv Giza 667 (more drought-sensitive) the Chl a/b ratio decreased significantly under drought conditions (Table 2), indicating that drought affected the size of the light-harvesting antenna. However, in cv Giza 40 (more drought-tolerant) the Chl a/b ratio increased with drought treatment. The change in the Chl a/b ratio is used as an indicator for relative photosystem stoichiometry (Pfannschmidt et al., 1999). Under control conditions, the Chl a/b ratio was comparatively higher in cv Giza 667 than in cv Giza 40, suggesting that the light-harvesting antenna was smaller in Giza 667 than in Giza 40. The question of whether the change in the size of the light-harvesting antenna is a useful indicator of the drought tolerance of plant species and genotypes will require further investigation.

The accumulation of soluble sugars and free amino acids, including proline, protects the cell under stress by balancing the osmotic strength of the cytosol with that of the vacuole and the external environment (Hellebust, 1976; Greenway and Munns, 1980; Gadallah, 1999). Besides their role as cytosolic osmotica these solutes may interact with cellular macromolecules such as enzymes and stabilize the structure of such macromolecules (Rhodes, 1987; Smirnoff and Cumbes, 1989; Jain et al., 2001). In the present study, the greater accumulation of osmolytes in the drought-tolerant cv Giza 40 compared with the drought-sensitive cv Giza 667 was associated with the greater accumulation of soluble proteins (Table 3). A direct consequence of higher osmolyte concentration in drought-tolerant cv Giza 40 is the comparatively higher water retaining capacity, as reflected by RWC (Table 2) and the more efficient antioxidant enzyme activity. This is confirmed by the work of Garg et al. (2001), who found higher contents of soluble sugars, free amino acids and proline in the drought-tolerant genotype CZM-32-E than in sensitive genotypes of moth bean. Türkan et al. (2005) also found higher proline content in drought-tolerant *Phaseolus acutifolius* under PEG-induced water deficit, while Sairam et al. (2002) observed higher soluble sugars and proline content in a tolerant wheat genotype (Kharchia 65) under salt stress.

In conclusion, based on the data of dry mass, RWC and the photosynthetically active pigments, it is clear that *V. faba* cv Giza 40 is more tolerant to drought stress than *V. faba* cv Giza 667. It is possible that the better resistance to drought of cv Giza 40 was related to its ability to maintain the higher activity of antioxidant enzymes such as CAT and POX, resulting in lower H_2O_2 production and lipid peroxidation and higher membrane stability. A higher osmolyte concentration (soluble sugars and free amino acids, especially proline) is important for osmoregulation, which is reflected in higher RWC and the stabilization of essential enzyme proteins such as CAT and POX, resulting in higher activity in cv Giza 40 under drought stress. Finally, the selection of *Vicia faba* cultivars with genetic traits such as greater antioxidant activity and osmolyte accumulation might be useful in improving the adaptive responses of *Vicia faba* to water stress.

References

Acar, O., Türkan, I., Özdemir, F. (2001): Superoxide dismutase and peroxidase activities in drought sensitive and resistant barley (*Hordeum vulgare* L.) varieties. *Acta Physiol. Plant.*, **3**, 351–356.

Ain-Lhout, F., Zunzunegui, M., Diaz Barradas, M. C., Tirado, R., Clavijo, A., Garcia Novo, F. (2001): Comparison of proline accumulation in two Mediterranean shrubs subjected to natural and experimental water deficit. *Plant Soil*, **230**, 175–183.

Barathi, P., Sundar, D., Reddy, R. (2001): Changes in mulberry leaf metabolism in response to water stress. *Biol. Plant.*, **44**, 83–87.

Bates, L. S., Waldern, R. P., Teare, I. D. (1973): Rapid determination of free proline for water-stress studies. *Plant Soil*, **39**, 205–207.

Bayuelo-Jimenez, J. S., Craig, G., Lynch, J. P. (2002): Salinity tolerance of *Phaseolus* species during germination and early seedling growth. *Crop Sci.*, **42**, 1584–1594.

Becana, M., Moran, J. F., Iturbe-Ormaetxe, I. (1998): Iron dependent oxygen free radical generation in plants subjected to environmental stresses: toxicity and antioxidants. *Plant Soil*, **201**, 137–147.

Bor, M., Özdemir, F., Türkan, I. (2003): The effect of salt stress on lipid peroxidation and antioxidants in leaves of sugar beet *Beta vulgaris* L. and wild beet *Beta maritima* L. *Plant Sci.*, **164**, 77–84.

Chance, B., Maehly, A. C. (1955): Assay of catalases and peroxidases. *Method Enzymol.*, **2**, 764–775.

Claussen, W. (2005): Proline as measure of stress in tomato plants. *Plant Sci.*, **168**, 241–248.

Dichio, B., Xiloyannis, C., Angelopoulos, K., Nuzzo, V., Bufo, S. A., Celano, G. (2003): Drought-induced variations of water relations parameters in *Olea europaea*. *Plant Soil*, **257**, 381–389.

Egert, M., Tevini, M. (2002): Influence of drought on some physiological parameters symptomatic for oxidative stress in leaves of chives (*Allium schoenopasum*). *Environ. Exp. Bot.*, **48**, 43–49.

El-Tayeb, M. A., Hassanein, A. M. (2000): Germination, seedling growth, some organic solutes and peroxidase expression of different *Vicia faba* lines as influenced by water stress. *Acta Agron. Hung.*, **48**, 11–20.

Fales, F. W. (1951): The assimilation and degradation of carbohydrates of yeast cells. *J. Biol. Chem.*, **193**, 113–118.

Fu, J., Huang, B. (2001): Involvement of antioxidants and lipid peroxidation in the adaptation of two cool-season grasses to localized drought stress. *Environ. Exp. Bot.*, **45**, 105–114.

Gadallah, M. A. A. (1999): Effect of proline and glycine betaine on *Vicia faba* responses to salt stress. *Biol. Plant.*, **42**, 247–249.

García-Valenzuela, X., García-Moya, E., Rascón-Cruz, Q., Herrera-Estrella, L., Aguado-Santacruz, G. A. (2005): Chlorophyll accumulation is enhanced by osmotic stress in graminaceous chlorophyllic cells. *J. Plant Physiol.*, **162**, 650–661.

Grag, B. K., Kathju, S., Burman, U. (2001): Influence of water stress on water relations, photosynthetic parameters and nitrogen metabolism of moth bean genotypes. *Biol. Plant.*, **44**, 289–292.

Girousse, C., Bournitrateville, R., Bonnemain, J. L. (1996): Water deficit-induced changes in concentration in proline and some other amino acids in phloem sap of alfalfa. *Plant Physiol.*, **111**, 109–113.

Greenway, H., Munns, R. (1980): Mechanism of salt tolerance in non halophytes. *Ann. Rev. Plant Physiol.*, **31**, 149–190.

Hellebust, J. A. (1976): Osmoregulation. *Ann. Rev. Plant Physiol.*, **27**, 485–505.

Hoagland, D. R., Arnon, D. I. (1950): The water culture method for growing plants without soil. *California Agri. Exp. Sta. Circ.*, **347**, p. 32.

Imlay, J. A., Linn, S. (1988): DNA damage and oxygen radical toxicity. *Science*, **240**, 1302–1309.

Jain, M., Mathur, G., Koul, S., Sarin, N. B. (2001): Ameliorative effects of proline on salt stress induced lipid peroxidation in cell lines of groundnut (*Arachis hypogaea* L.). *Plant Cell Rep.*, **20**, 463–468.

Kirnak, H., Kaya, C., Tas, I., Higgs, D. (2001): The influence of water deficit on vegetative growth, physiology, fruit yield and quality in eggplants. *Bulg. J. Plant Physiol.*, **27**, 34–46.

Kocheva, K., Georgiev, G. (2003): Evaluation of the reaction of two contrasting barley (*Hordeum vulgare* L.) cultivars in response to osmotic stress with PEG 6000. *Bulg. J. Plant Physiol.*, Special Issue, 290–294.

Le Thiec, A., Manninen, S. (2003): Ozone and water deficit reduced growth of Aleppo pine seedlings. *Plant Physiol. Biochem.*, **4**, 55–63.

Levy, D. (1983): Water deficit enhancement of proline and amino nitrogen accumulation in potato plants and its association with susceptibility to drought. *Physiol. Plant.*, **57**, 169–173.

Loggini, B., Scartazza, A., Brugnoli, E., Navari-Izzo, F. (1999): Antioxidative defense system, pigment composition and photosynthetic efficiency in two wheat cultivars subjected to drought. *Plant Physiol.*, **119**, 1091–1099.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., Randall, R. J. (1951): Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

Metzner, H., Rau, H., Senger, H. (1965): Untersuchungen zur Synchronisierbarkeit einzelner Pigmentmangel mutanten von *Chlorella*. *Planta*, **65**, 186–194.

Moore, S., Stein, W. (1948): Photometric ninhydrin method for use in the chromatography of amino acids. *J. Biol. Chem.*, **176**, 367–388.

Munné-Bosch, S., Alegre, L. (2000): The significance of β-carotene, α-tocopherol and the xanthophyll cycle in droughted *Melissa officinalis* plants. *Aust. J. Plant Physiol.*, **27**, 139–146.

Parker, W. C., Pallardy, S. G. (1985): Genotypic variation in tissue water relations of leaves and roots of black walnut (*Juglans nigra*) seedlings. *Physiol. Plant.*, **64**, 105–110.

Patel, J. A., Vora, A. B. (1984): Free proline accumulation in drought-stressed plants. *Physiol. Plant.*, **20**, 230–232.

Pfannschmidt, T., Nilsson, A., Allen, J. F. (1999): Photosynthetic control of chloroplast gene expression. *Nature*, **397**, 625–628.

Reddy, R. A., Chaitanya, K. V., Jutur, P. P., Sumithra, K. (2004): Differential antioxidative responses to water stress among five mulberry (*Morus alba* L.) cultivars. *Environ. Exp. Bot.*, **52**, 33–42.

Rhodes, D. (1987): Metabolic responses to stress. pp. 201–241. In: Stumpf, P. K., Priess, J. (eds.), *The Biochemistry of Plants*, Vol. 12. Academic Press, San Deigo, CA.

Sairam, R. K., Rao, K. V., Srivastava, G. C. (2002): Differential response of wheat genotypes to long term salinity stress in relation to oxidative stress, antioxidant activity and osmolyte concentration. *Plant Sci.*, **163**, 1037–1046.

Sairam, R. K., Srivastava, G. C. (2001): Water stress tolerance of wheat (*Triticum aestivum* L.): Variations in hydrogen peroxide accumulation and antioxidant activity in tolerant and susceptible genotypes. *J. Agron. Crop Sci.*, **186**, 63–70.

Sánchez-Blanco, M. J., Rodríguez, P., Morales, M. A., Ortuo, M. F., Torrecillas, A. (2002): Comparative growth and water relations of *Citrus albidus* and *Citrus monspeliensis* plants during water deficit conditions and recovery. *Plant Sci.*, **162**, 107–113.

Savé, R., Biel, C., Domingo, R., Ruiz-Sanchez, M. C., Torrecillas, A. (1995): Some physiological and morphological characteristics of citrus plants for drought resistance. *Plant Sci.*, **110**, 167–172.

Schmidhalter, U., Oertli, J. J. (1991): Germination and seedling growth of carrots under salinity and moisture stress. *Plant Soil*, **132**, 243–251.

Schütz, W., Milberg, P., Lamont, B. (2002): Germination requirements and seedling responses to water availability and soil type in four eucalypt species. *Acta Oecol.*, **23**, 23–30.

Sestak, Z., Vaclavick, J. (1965): pp. 210–216. In: Slavik, B. (ed.), *Water Stress in Plants*. Czech. Acad. Sci., Prague.

Sgherri, C. L., Maffei, M., Navari-Izzo, F. (2000): Antioxidative enzymes in wheat subjected to increasing water deficit and rewatering. *J. Plant Physiol.*, **157**, 273–279.

Shaddad, M. A., El-Tayeb, M. A. (1990): Interactive effects of soil moisture content and hormonal treatment on dry matter and pigments content of some crop plants. *Acta Agron. Hung.,* **39**, 49–57.

Smirnoff, N., Cumbes, Q. T. (1989): Hydroxyl radicals scavenging activity of compatible isolates. *Phytochemistry*, **28**, 1057–1060.

Smirnoff, N. (1993): The role of active oxygen species in response of plants to water deficit and desiccation. *New Phytol.*, **125**, 57–58.

Torrecillas, A., Guillaume, C., Alarcón, J. J., Ruiz-Sánchez, M. C. (1995): Water relations of two tomato species under water stress and recovery. *Plant Sci.,* **105**, 169–176.

Türkan, I., Bor, M., Özdemir, F., Koca, H. (2005): Differential response of lipid peroxidation and antioxidants in the leaves of drought-tolerant *P. acutifolius* Gray and drought-sensitive *P. vulgaris* L. subjected to polyethylene glycol mediated water stress. *Plant Sci.*, **168**, 223–231.

Vyas, S. P., Kathju, B. K., Garg, B. K., Lahiri, A. N. (1985): Performance and metabolic alterations in *Sesamum indicum* L. under different intensities of water stress. *Ann. Bot.*, **56**, 323–331.

Whetherley, P. E. (1950): Studies in the water relations of cotton plants. I. The field measurement of water deficit in leaves. *New Phytol.*, **49**, 81–87.

Yu, Z., Rengel, Q. (1999): Drought and salinity differentially influence activities of superoxide dismutase in narrow leafed lupines. *Plant Sci.,* **141**, 1–11.

Zaho, S. J., Xu, C. C., Zou, Q. (1994): Improvements of the method for measurement of malondialdehyde in plant tissue. *Plant Physiol. Commun.*, **30**, 207–210.

Zrenner, R., Stitt, M. (1991): Comparison of the effect of rapidly and gradually developing water stress on carbohydrate metabolism in spinach leaves. *Plant Cell Environ.,* **14**, 939–946.

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MICROCLIMATE AND TRANSPIRATION OF REEDBEDS ON LAKESHORES WITH CHANGING WATER LEVELS

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Changes in the microclimate and transpiration of reedbeds on the shores of Lake Balaton, some still standing in water and some from which the water has receded, were examined in Keszthely Bay during the 2003 vegetation cycle, after canopy closure. Microclimate data were recorded using combined sensors connected to a data-collector. Ten-minute means calculated from data recorded every two seconds were used as the basis of comparison. Transpiration was quantified using the shoot mass loss method. The leaf area index of reeds growing on dry areas was greater than that of plants rooted in the lake. This difference was moderate at first, but increased greatly during the vegetative phase, and could be attributed primarily to differences in plant density and leaf size, and to the effect of waves. Among the components of the microclimate, the humidity within the stand was 8–20% greater for plants growing in water, irrespective of the weather and the development stage. The daily mean air temperature was lower in the dry stand, and exhibited considerable daily fluctuations. The mean daily sum of transpiration, averaged over three sample days, was 16.5% lower in the dry stand. The plot of daily changes in transpiration exhibited two peaks for the dry stand and one for the wet stand.

Key words: microclimate, reed architecture, water loss

Introduction

Reeds are known to be extremely sensitive to changes in the environment and to exhibit morphological deviations depending on the habitat (Zheng et al., 1999; Chen et al., 2004) and on transpiration (Herbst and Kappen, 1999; Sanchez-Carrillo et al., 2004). Reeds growing in a water-covered environment have a larger number of stomata and these are also more deeply embedded into the epidermis. Reeds growing in dry habitats compensate for the smaller number of stomata by increasing the size of the openings and by causing an increase in the transpiration surface. A wide-ranging summary of changes in the composition and size of reeds in Lake Balaton was published by Virág (1998).

This work was mainly focused on the ecology of reed stands. In Hungary, earlier investigations on water loss in reedbeds were carried out mainly under artificial conditions using lysimeters, during the 1970s and 1980s (Szilágyi, 1974; Walkovszky, 1980; Baranyi, 1985). Little information is available about *in situ* transpiration measurements on reed canopies in the surroundings of the Balaton and Fertő lakes. One of the aims of the present work was to measure reed transpiration on the lakeshore in an untouched environment. These results may differ from measurements made in lysimeter growth chambers.

By contrast to morphological modifications, little attention was paid to the responses of plants to changes in the environment, especially in the microenvironment. The slightest change in the microclimate is capable of influencing life processes, and transpiration is particularly sensitive to such changes. Reeds have broad ecological valence, as confirmed by their widespread occurrence, so it is difficult to define the environmental limits that might inhibit their spread and satisfactory development. The aim of the present work was thus to detect microclimatic changes caused by differences in the water level in the reedbed and to quantify the resulting change in transpiration. It is frequently observed in nature that reedbeds undergo significant changes in water supply from time to time, becoming temporarily dry or completely inundated with water.

Materials and methods

Observations were made in the vegetation season of 2003 on two natural reedbeds *(Phragmites australis)* near Fenékpuszta on the shores of Keszthely Bay, Lake Balaton, one of which was standing in 30–50 cm of water, the other 30–40 metres from the waterline, under dry conditions with no water cover.

The shoot mass loss method (Sutcliffe, 1982) was used to calculate the transpiration values, taking measurements on shoot mass at hourly intervals between 8 am and 5 pm in three replications using a portable, battery-operated digital balance. Linear regression was employed to determine the intensity of transpiration and a straight line was fitted to the declining shoot mass data registered at 1-hour intervals. The transpiration data were compared on the basis of the equations describing the curves, after which the intensity of transpiration was calculated in terms of 1 m^2 leaf area. The total daily transpiration was given by the integral of the curves.

The area of the cut shoot sections and the leaf area in the different treatments were recorded using a LI-COR LI-3000A portable, automatic planimeter. Detailed data on the leaf length, width and maximum width of 20 plants per sample were used to determine the vertical distribution of the leaf area.

The choice of methods meant that measurements could only be taken at certain stages of plant development, thus restricting the choice of sample days. The greatest changes in the microclimate can also be observed in closed, well-developed stands, particularly after dry periods, so data collection was begun in July, at the same time as the stand temperature measurements, and was continued until September.

Daily changes in the components of the microclimate were recorded using combined sensors capable of measuring global irradiation, relative air humidity and air temperature, linked to meteorological data collectors of the LI-COR 1000 type. Trends in each component were traced using 10-minute means of data taken every two seconds. The frequent sampling was necessary due to the extreme variability in the microclimate. With the exception of the radiation meter, the sensors were placed within the stand, in the standard manner, at a height of 1.5 m from the ground. The radiation meter was sited 2 m above the stand.

Results

Description of plant parameters and macroclimate during the measurements

The mean height of the wet and dry stands was similar in July, but the wet stand had a leaf area index (LAI) of 4.3 compared with 5.1 for the dry stand. Even during the vegetative phase, the first leaf storeys of reeds standing in water were found approx. 20 cm above the water surface; this loss of leaves could be the result of continual wave motion. Although the height of the sample plants was the same, reeds in the dry stand developed only 12 leaf storeys, compared with 16 in the wet stand, so the internodes were shorter in the latter. During the vegetative phase the mean length of the leaves in the dry stand was 2–3 cm greater in the middle third of the plant than in the wet stand. This, combined with the greater plant density, contributed to the higher LAI of the dry stand.

In August plants from the two stands with the same number of leaf storeys exhibited a significant difference in plant height. While the height of the dry stand only increased by the length of the inflorescence (13.5 cm), reeds in the wet stand grew more than 50 cm. The plant density remained constant at the July level, but the LAI increased to 7.1 in the dry stand, while there was no significant change in that of reeds growing in water. The mean width of the leaves at the various levels was 3–5 cm greater in the dry stand, while the maximum leaf width was 0.5 cm greater than in the wet stand. This lack of change in the leaf area of the wet stand was probably due to the fact that the leaf loss caused by constant water lapping was greater than in July and was only partially compensated for by the formation of new leaves. By August the first leaf storey was 50–60 cm above the water level.

There was no change in plant density during the measurement period, the wet stand being thinner throughout the vegetation period, with 38 shoots m^{-2} compared with 55 shoots m^{-2} in the dry stand.

In 2003 the mean temperature was 1.6°C higher in July and 3.7°C higher in August than the mean over many years. These months were both considerably drier than usual, since there was 25.5% less rainfall than average, with extremely uneven distribution.

Characteristics of the microclimate in fully developed reedbeds

The growth and development of plant stands is greatly influenced by the immediate atmosphere through metabolic and energy exchange processes. The relationship between living organisms and their environment is an interaction, as the result of which plant stands have a special microclimate, formed within the given macroclimate by a whole series of processes taking place between the ground surface and the atmosphere.

The effect of different water supply levels on the microclimate can be clearly detected during lengthy dry periods. The equilibrating effect of rainfall and wind on the microclimate is illustrated by the daily changes in meteorological factors on a wet, overcast day (Fig. 1).

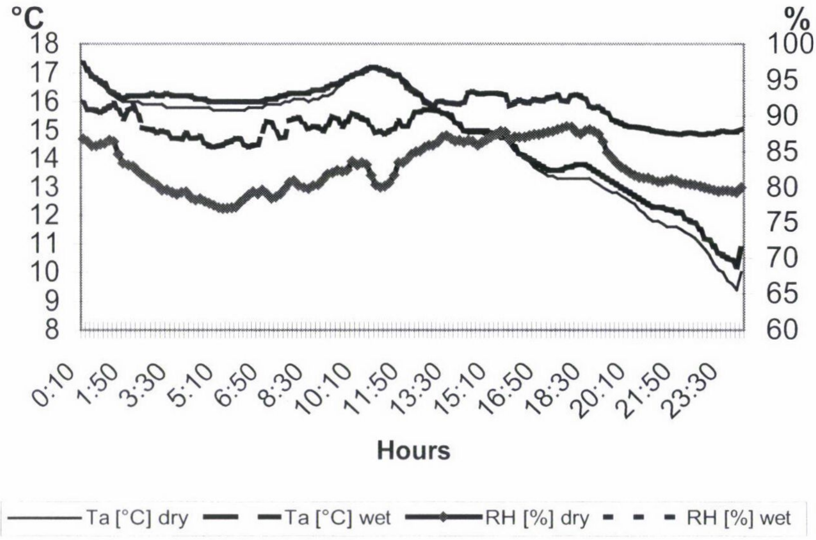

Fig. 1. Daily changes in microclimate components on a rainy, overcast day (31 August, 2003). Global radiation: 260 W m^{-2}; daily mean air temperature (Ta): 15.2°C; daily mean relative humidity (RH): 78%; daily sum of precipitation: 15.5 mm

Compared with the data recorded on cloudless days a moderate difference was only observed in the relative humidity, but this was true of all the sampling days. The difference in air temperature caused by the differing water supplies became negligible when the sky was cloudy.

The microclimate data for cloudless and windless days in July and August were recorded on the days when transpiration was investigated (Fig. 2a, b).

The diurnal changes in air temperature exhibited a slight difference for the two habitats. The direction of this change was the same throughout the measurement period, but the extent and time of occurrence differed for stands in the vegetative and generative phases. During the night and early morning, when radiation heat loss is predominant the air temperature was greater within the aquatic plant stand (Fig. 2a). When the sun was high in the sky, however, the air temperature within the dry-standing reedbed was higher, until 3–4 pm in July and for a little longer, until 7 pm in August. The difference between the 10-min means may be as great as 1.5–2.0°C. This can be attributed to the different radiation and heat characteristics of water and soil, as the result of which the water surface supplies more energy than the soil during the period when reflected heat is predominant, while at a high solar angle the soil warms up to a greater extent, leading to a higher temperature in the dry stand. Water and soil have different specific heats. The water mass stores heat well, so it acts as a cooling medium by day and a heating medium by night, thus reducing the fluctuation in the values recorded for reeds growing in an aqueous habitat. The 0.5–1.5°C difference in the daily means in July was reduced to about a third in August, but plants standing in water were always warmer. The air temperature recorded in stands with limited water supplies exhibited extreme variation, since the fluctuation value on cloudless days was 12–19°C, while this was only 9–15°C for reeds growing in water.

The relative air humidity was always greater in the wet stands, regardless of the weather, the development phase and the measuring date (Fig. 2b). The difference in the daily changes was sometimes more than 20%. In July the mean daily relative humidity of the dry stand was 13–18% lower than that of the wet stand, while this difference, like that in the air temperature, was considerably smaller in August (5–8%).

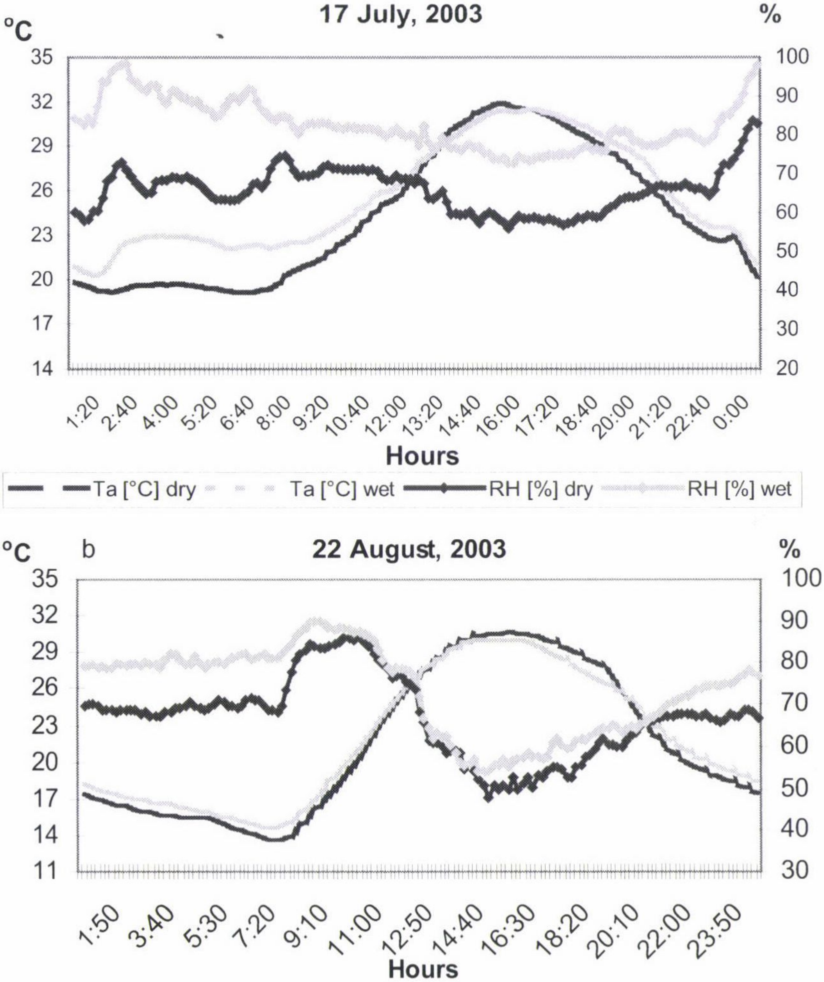

Fig. 2. Diurnal variation in air temperatures and relative humidity during clear, calm days in 2003 Global radiation: a. 640, b. 625 W m^{-2}; daily mean air temperature: a. 26.2, b. 24.9°C; daily mean relative humidity: a. 65, b. 60%

Trends in daily transpiration sum of closed reedbeds

Three sample days, providing a good representation of the weather during the whole vegetation period, were chosen to illustrate the results obtained for transpiration. The first, July 15th, is characteristic of the long period of moderately hot, dry weather in the first half of the month, when the daily rainfall sum was never more than 4 mm. The much hotter weather experienced in the second half of July is represented by the second sample day, July 17th, when there was somewhat more rainfall, but still far less than the climatic average. In the whole of August 2003 the weather was very hot, record temperatures being measured on several days, and this period was also extremely dry, as demonstrated by the total rainfall of less than 10 mm. The transpiration during this dry, hot period is represented by the third sample day, August 22nd.

After the stands closed, in the cooler first half of July 2003, the daily mean water loss per unit leaf area was 4–5 mm in both reedbeds (Table 1). The daily mean transpiration of plants standing in water was only 15.4% greater than that of dry-standing reeds.

The warmer weather in the second half of July increased the daily transpiration sum by 1–2 mm per m^2, the increase being more intense in the wet stand (30.8%) and more moderate in the dry stand (19.6%). During the hot, dry August the plant transpiration values were even higher, but the difference between the two water supply treatments dropped to 10.9%. On these hot August days the transpiration of plants standing in Lake Balaton was 8 mm or more per unit area on all the sample days (with a value of 8.71 mm on August 22nd).

Although the daily mean water losses of dry reedbeds were very close to the average evapotranspiration of reed stands grown in a lysimeter, no comparison was made because of differences in their environments. Reeds grown in a lysimeter provide evapotranspiration values, while *in situ* measurements provide transpiration data alone. The lysimeter would thus represent a third water treatment.

Discussion

On overcast or windy sampling days there was little or no difference between the two different stands for either transpiration or the components of the stand microclimate.

Table 1

Major characteristics of transpiration in closed reed stands on sample days in 2003

Characteristic parameters	15 July 2003		17 July 2003		22 August 2003	
	Dry stand	Wet stand	Dry stand	Wet stand	Dry stand	Wet stand
Daily mean water loss$^+$	7.12	8.31	8.98	11.38	14.21	15.67
SD (Standard Deviation)	±1.68	±2.49	±0.208	±2.85	±2.65	±3.58
Water loss from the whole canopy*	4.1	4.7	4.99	6.41	7.81	8.71

$^+$g m^{-2}; *mm m^{-2}

It should be noted here that the height of the leaf storey closest to the ground or the water was not the same in the two stands (Fig. 3). The height of the first green leaf on plants growing in the water-covered environment was about 20 cm above the water level in July and 50–60 cm in August, while on plants growing on dry land the average height above the ground was 76 cm. The greater leaf area of plants in the dry treatment could be attributed not only to the greater plant density, but also to the larger leaves formed in the middle part of the plants. It was surprising to find that, despite the lower plant density, the plants in the aqueous habitat had somewhat smaller leaves, though this did not prevent the stand from closing.

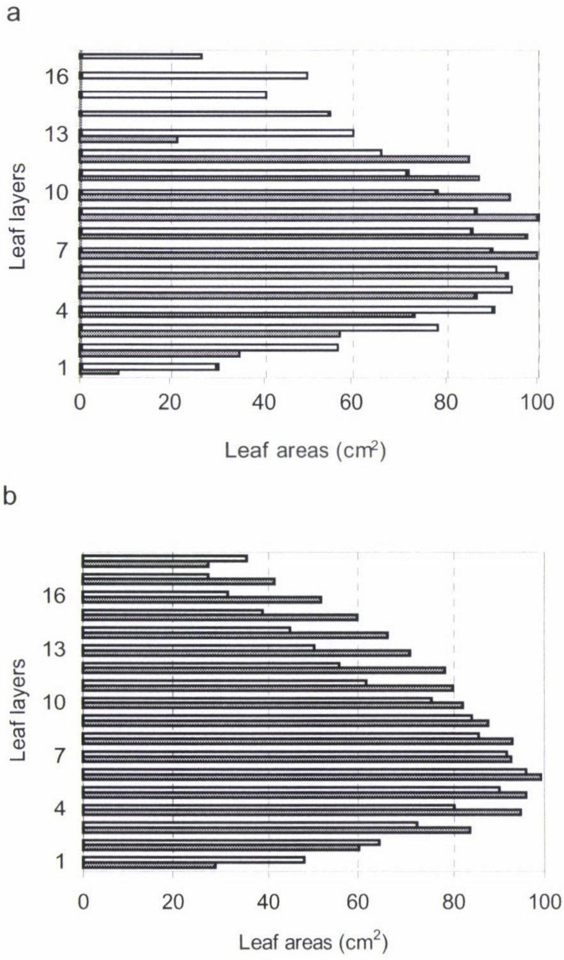

Fig. 3. Vertical distribution of leaf areas at different levels of plant height during the vegetative a) and generative (b) phases of development. Empty columns designate reeds standing in water, and grey ones plants developing under "dry" conditions

The dying off of leaves in the lower storeys began in July for plants growing in water, and continued practically throughout August, while in the dry stand dying off began later. As a consequence, the shorter reeds growing on dry land had the same number of leaf storeys by August as the much taller plants growing in water.

The difference in total daily transpiration due to the diverse water supplies was moderate in July, but increased to several mm/m^2 in August, as a result of the long heat-wave. The transpiration values recorded for reeds standing in the lake are in agreement with those reported by Burba et al. (1999) during a similar period in an aqueous habitat. Not only the magnitude of the transpiration, but the shape of the curve describing daily changes in transpiration also depended on the water supplies. The transpiration graph for plants standing in water was a regular curve with a single peak, exhibiting a maximum between noon and 3 pm (Anda and Boldizsar, 2005). The intensity of transpiration fluctuated greatly in the dry stand and there were two peaks, one between 11 am and noon, the other between 3 and 4 pm. At a high solar angle, due to the low air humidity and high temperature, plants in the dry habitat close their stomata to reduce transpiration water loss, thus allowing them to use their water reserves to maintain turgor. In this way they are able to prevent increased water loss at high radiation intensity, which plants in dry habitats would find difficult to replace in the afternoon. As soon as the radiation and air temperature drop to the required level, the stomata open and transpiration is resumed. This phenomenon cannot be observed in reed stands growing in water, since they always have an unlimited water supply for the purposes of transpiration. This finding is confirmed by trends in relative air humidity, which was 10–15% lower in the early morning hours on sampling days in the dry stand than for plants growing in a water-covered environment. The drier the air the more moisture it is able to absorb, so the more intensely it extracts water from its environment, in the present case from the plant stand. Throughout the observation period, irrespective of the weather, the air humidity was always greater in reeds growing in water.

Acknowledgements

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References

Anda, A., Boldizsar, A. (2005): Transpiration and plant surface temperatures of reedbeds with different watering levels. *Georgikon Agric.*, **8**, 39–52.

Baranyi, S. (1985): A Velencei tó havi párolgásának becslése parti kád-mérések alapján. (Evaluation of the monthly transpiration of Lake Velencei according to data from coastal measuring vats.) *Hydrological Szemelvények*, **4**, 578–589.

Burba, G. G., Verma, S. B., Kim, J. (1999): Surface energy fluxes of *Phragmites australis* in a prairie wetland. *Agr. Forest Meteorol.*, **94**, 31–51.

Chen, Y. N., Li, W. H., Chen, Y. P., Zhang, H. F., Zhuang, L. (2004): Physiological response of natural plants to the change of groundwater level in the lower reaches of Tarim River, Xinjiang. *Prog. Nat. Sci.*, **14**, 975–983.

Herbst, M., Kappen, L. (1999): The ratio of transpiration versus evaporation in a reed belt as influenced by weather conditions. *Aquat. Bot.*, **63**, 112–125.

Sanchez-Carillo, S., Angeler, D. A., Sanchez-Andrez, R., Alvarez-Cobelas, M., Garatuze-Payan, J. (2004): Evapotranspiration in semi-arid wetlands: relationships between inundation and the macrophyte-cover: open-water ratio. *Adv. Water Resour.*, **27**, 643–655.

Sutcliffe, J. (1982): *Növények és a víz.* (Plants and Water.) Mezőgazdasági Kiadó, Budapest.

Szilágyi, T. (1974): A fertő tavi nád biológiai és gazdasági (ipari) érésének időjárási vonatkozásai. (Meteorological aspects of the biological and economic maturation of reed crops on Lake Fertő.) *Az OMSZ Hivatalos Közleményei*, **44**, 177–180.

Virág, Á. (1998): *A Balaton múltja és jelene.* (The Past and Present of Lake Balaton.) Vízügyi Múzeum, Budapest. pp. 603–640.

Walkovszky, A. (1980): Csapadék-intercepció a Fertő tavi nádasban. (Precipitation interception in the reeds of Lake Fertő.) *Az OMSZ Hivatalos Közleményei*, **50**, 200–212.

Zheng, W. J., Wang, S., Zhang, C. L. (1999): A study on the leaf structure of four reed ecotypes. *Acta Bot. Sin.*, **41**, 565–580.

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COMPARATIVE ANALYSIS OF STRESS TOLERANCE IN *Aegilops* ACCESSIONS AND *Triticum* WHEAT VARIETIES TO DETECT DIFFERENT DROUGHT TOLERANCE STRATEGIES

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The drought stress tolerance of three accessions of *Aegilops biuncialis* Vis. (Ae225, Ae550 and Ae1050) and two varieties of *Triticum aestivum* L. (Sakha and Cappelle Desprez) was compared. The activity of superoxide dismutase (SOD) isoenzymes, which reflects the intensity of oxidative stress, changes in the malonic dialdehyde (MDA) content, formed during the lipid peroxidation induced by stress situations, and the inducibility of electron removal systems appearing as an alternative to CO_2 fixation were chosen for the present investigations. Drought stress was simulated using polyethylene glycol (PEG). The order of drought stress tolerance obtained correlated well with the original habitats of the varieties. The present results provide a clear illustration of the fact that tolerant varieties respond differently for the parameters tested, suggesting that their resistance can be attributed to different mechanisms.

Abbreviations: CuZnSOD=superoxide dismutase isoform with Cu and Zn cofactor metals, MnSOD and FeSOD=superoxide dismutase isoform with Mn and Fe cofactor metals, PVP25= polyvinyl pyrrolidone 25, MDA=malonic dialdehyde, PEG=polyethylene glycol, TCA=trichloro acetic acid, TBA=thiobarbituric acid, $\Delta F=F_m-F_s$, F_m=maximal fluorescence yield, F_s=fluorescence yield in steady state

Key words: drought stress, PEG, lipid peroxidation, MDA, oxidative stress, SOD, *Aegilops biuncialis* Vis., *Triticum aestivum* L., drought resistance strategies

Introduction

Under normal circumstances, O_2 is reduced to water by 4 electrons as part of the mitochondrial electron transport chain of the aerobic catabolic metabolism. In various stress situations, complete reduction is hindered by the acceleration of energy-demanding processes, the limited electron permeability of the transport chain and the lack of oxidized coenzymes, so superoxide anions are generated. When light absorption exceeds the capacity of the photosynthetic apparatus, singlet oxygen and superoxide anion radicals are also generated by various mechanisms in the electron transport chain of chloroplasts. The isoenzymes of superoxide dismutase (SOD) play a vital role in the elimination of these reactive oxygen species (ROS), a role which is essential for living organisms, as ROS cause various degrees of damage in cells (Fridovich, 1986), including the degradation of the core complex and membrane structures in PSII, chromosome aberrations and, in serious cases, cell death.

Even under physiological conditions, superoxide dismutase (SOD) isoenzymes are relatively active in each plant compartment. The majority of the total activity is due to the functioning of the CuZnSOD isoform, which can be found in the cytoplasm and the chloroplasts. Isoenzymes containing Fe, located in the chloroplasts, and those of the MnSOD type, located in the mitochondria, are also active in green plant tissues (Harris et al., 1980). Under stress conditions, pronounced changes occur in the activity of these isoenzymes, reflecting the level of stress in the individual compartments (Borsania et al., 2001). One clearly perceptible physiological effect of drought stress treatment is a varying extent of stomatal closure, depending on the level of stress. One indirect consequence of long-term stomatal closure is a reduction in the efficiency of CO_2 fixation. This is why measurements were made on the photochemical efficiency of PSII ($\Delta F/F_m$') in an experiment where CO_2 fixation was inhibited. The values of $\Delta F/F_m$' in control and PEG-treated plants during drought stress treatments give a clear indication of plants which activate alternative electron removal mechanisms capable of eliminating the electrons accumulating in the course of inhibited photosynthetic electron transport and thus protecting the components of the transport chain from damage. Varieties with different levels of drought stress tolerance exhibited differences in their ability to activate alternative electron removal systems, thus giving a good illustration of one possible strategy involved in tolerance.

Malonic dialdehyde (MDA), an intermediary product arising during the peroxidative decomposition of lipids, is an easily measured marker of the oxidative stress induced by drought. Its physiological concentration is in the nanomolar range. Processes involving intensive lipid peroxidation, such as the decomposition and conversion of membranes as the result of oxidative stress, or morphogenetic changes, cause an increase in this concentration. In seedlings there are significant changes in the MDA level even under control conditions.

The three *Aegilops biuncialis* Vis. accessions included in the stress tolerance studies originated from habitats with very different mean rainfall values (AB1094: 225 mm, henceforth Ae225; AB 382: 550 mm, henceforth Ae550; AB 642: 1050 mm, henceforth Ae1050), while the *Triticum aestivum* L. variety Sakha, of Egyptian origin, is known to have good drought tolerance and the French variety Cappelle Desprez (henceforth Cappelle) is extremely drought-sensitive.

As expected, the stress tolerance of the *Aegilops* accessions, characterised by SOD isoenzyme activity and MDA content, was inversely proportional to the annual rainfall quantities in their habitats (Selote et al., 2004). The good tolerance of Sakha and the sensitivity of Cappelle were clearly reflected in the stress physiological parameters recorded. The MDA content measured in the tissues of different varieties confirmed the extent of drought tolerance indicated by the SOD isoenzyme activity. This was particularly obvious in the case of Ae550, which consistently exhibited the lowest level of changes in the MDA content in response to the treatment compared with the other varieties and accessions. This can probably be attributed to the outstanding salt tolerance reported in the literature for this variety (Molnár et al., 2004). In addition to the significantly low MDA content and changes in this content, the CO_2 fixation data also confirm the classification made on the basis of SOD isoenzyme activity. Based on the inhibition of CO_2 fixation, the SOD isoenzyme activity and the MDA content, the drought tolerance of the varieties and accessions decreased in the following order: Sakha, Ae550 > Ae225 > Ae1050 >> Cappelle.

Materials and methods

Wheat varieties, accessions and plant growth

The experiments were carried out using three *Aegilops biuncialis* Vis. accessions originating from habitats with very different mean annual rainfall values (Ae225, Ae550 and Ae1050), and two *Triticum aestivum* L. varieties, Sakha, of Egyptian origin, and the French variety Cappelle Desprez. Germinated seedlings of the *T. aestivum* and *Ae. biuncialis* accessions were grown in half-strength modified Hoagland nutrient solution (Nagy and Galiba, 1995) in a plant growth chamber (Conviron, Manitoba, Canada) with 12/12 h day/night cycles of 18/13°C for 7 d, followed by 20/18°C for 7 d, with 70% relative humidity and 200 $\mu mol\ m^{-2}\ s^{-1}$ light intensity (Tischner et al., 1997).

Drought stress treatment

After two weeks of growth the plants were subjected to increasing concentrations of polyethylene glycol (PEG6000, Sigma, St Louis, MO) in the nutrient solution (12, 15, 18 and 21 m/v%) for four weeks and samples of control and treated plants were taken weekly throughout the experiment. The treated plants were then transferred to control nutrient solution for recovery, and measurements were made after 3 and 7 days.

Fluorescence induction measurements

Fluorescence induction measurements were performed on intact and detached leaves pre-incubated in 30 mM glyceraldehyde for 2 h, using a PAM-2000 Chlorophyll Fluorometer (Walz, Effeltrich, Germany). The leaf samples were dark-adapted for 20 minutes prior to measurement. Actinic white light (200 $\mu mol\ m^{-2}\ s^{-1}$) was provided and the steady-state level of fluorescence (F_s) was determined by the measuring light (100 kHz, 1 $\mu mol\ m^{-2}\ s^{-1}$). The maximum fluorescence yield in the light-adapted state (F_m') was measured after applying a 0.7 s pulse of white light (2500 $\mu mol\ m^{-2}\ s^{-1}$). The effective quantum efficiency of photosystem II was calculated as $(F_m'-F_s)/F_m'(\Delta F/F_m')$.

Determination of enzyme activity; gel electrophoresis

For the analysis of superoxide dismutase isoenzymes, 100 mg fresh leaves were homogenised in 200 µl extraction buffer (Sörensen phosphate buffer 25 mM, pH 7.00, 1 mM EDTA, pH 8.00 and 20.0 g/l PVP25). The samples were centrifuged at 10,000 g for 15 min at 4°C. The supernatant was stored at –70°C. The SOD isoenzyme forms were separated using gradient SDS gel electrophoresis (9–12%). The activity of active SOD isoforms was detected using the method of Beauchamp and Fridovich (1971). The samples were not subjected to heat treatment before being placed on the gel. Densitometry and subsequent image processing were used for the qualitative and quantitative analysis of the SOD isoforms and their activity.

Determination of MDA content

Fresh plant material (100 mg) was mixed with 500 µl 0.1% trichloro acetic acid (TCA) and homogenised. The samples were kept on ice until centrifugation at 10,000 g for 10 min at 4°C. One ml MDA reagent (20.0% TCA solution containing 1.0% thiobarbituric acid, TBA) was added to 250 µl supernatant, and after 30 min incubation at 100°C the samples were cooled and examined spectrophotometrically at room temperature using the method of Heath and Packer (1968).

Statistics

Three or five parallel measurements were made in each experiment. For each measurement the sample group contained three parallels. The calculation of the percentage deviation of the measured values and the fitting of the curves were carried out using Origin 5.0 and Microsoft Excel software.

Results and discussion

In the control plants there tended to be a slow increase in SOD activity with time, except in varieties and accessions with poor drought tolerance, where there was a continuous drop in activity (Fig. 1).

The drought stress tolerance of *Aegilops* accessions from different habitats (Ae225, Ae550, Ae1050) proved to be inversely proportional to the annual rainfall quantities in their original habitats, as reflected in changes in SOD isoenzyme activity, in the MDA content of leaf tissues and in the efficiency of CO_2 fixation: Ae550, Ae225 > Ae1050. The activity of the MnSOD and CuZnSOD isoforms was relatively constant during the stress treatments (Ae225, Ae1050), rising slightly during recovery (Figs. 2, 3A). In the recovery state the profile and range of the SOD enzyme activity were similar in Sakha and Ae550 and very different to those in the other plants (Fig. 3B). This all suggests that, after a certain point, this variety is unable to control increasing oxidative stress, or that the necessary synthetic processes or the demand for reductants exceed its potential. The situation is similar for the variety Cappelle, which had the poorest drought tolerance of all the genotypes tested and where SOD activity decreased with time in the control plants (Fig. 1). At the same time, among the two most drought-tolerant varieties, Sakha exhibited a continuous increase in SOD activity (Fig. 1). Of all the active isoforms, the CuZnSOD isoforms were dominant both in the control and in the treated variants (Figs. 1, 2).

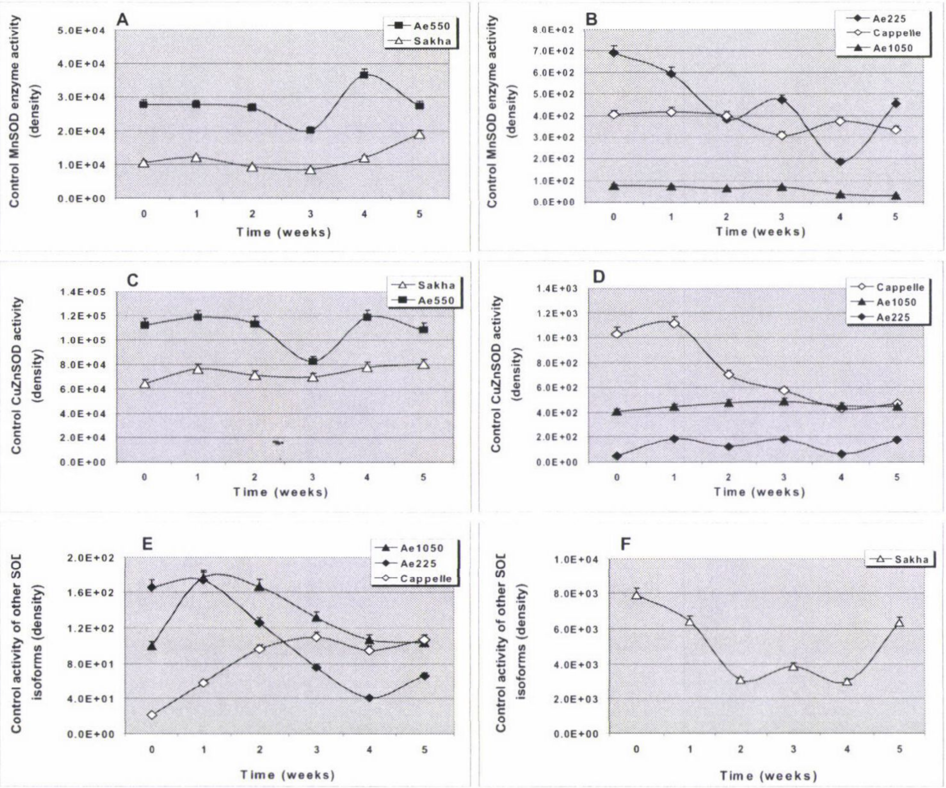

Fig. 1. SOD isoenzyme activity in control leaves as a function of the growth period (weeks). *Ae. biuncialis* genotypes and *T. aestivum* varieties were grown under control conditions

The only exception was Ae225, where MnSOD activity was dominant under control conditions, with low CuZnSOD activity, while in the treatments the activity of both MnSOD and CuZnSOD declined and the activity of isoforms with low molecular weights became dominant (Figs. 1, 2). When the SOD activity of the variety Ae225, which had excellent drought tolerance, was compared with that of Ae550 and Sakha, which also had a substantial level of tolerance, it was found that the activity of SOD isoforms in Ae550 and Sakha was two orders of magnitude greater than that of both Ae225 and of varieties and accessions with poorer tolerance (Ae1050, Cappelle) (Figs. 1, 2). The three varieties with good drought tolerance (Sakha, Ae225, Ae550) thus exhibited different isoform dominance within the total activity; in Ae225 MnSOD was dominant under control conditions and isoforms with low molecular weights in the treatments, while in Sakha and Ae550 the activity of CuZnSOD isoforms was dominant (Figs. 1, 2).

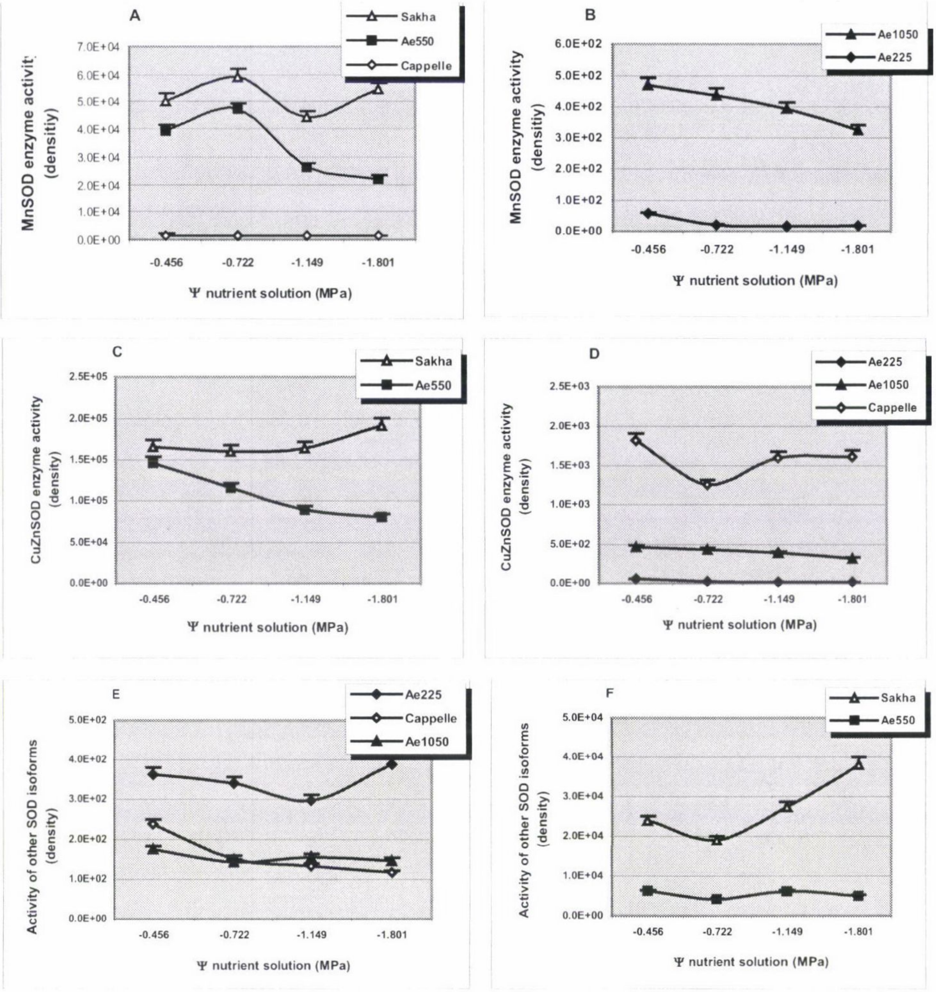

Fig. 2. Change in the SOD activity of PEG-stressed leaves as a function of the water potential of the nutrient solution (MPa). Measurements were made at the end of the 7-day treatment periods (water potential of the nutrient solution was as follows: 0–7 days: –0.46 MPa; 7–14 days: –0.72 MPa; 14–21 days: –1.15 MPa; 21–28 days: –1.80 MPa)

With respect to the MDA content of the leaves, the level in Sakha leaves was consistently found to be around 2 nmol/g fresh weight, a value close to the physiological level, with the exception of two samples (Fig. 4). The highest values (> 5 nmol/g fresh weight) were recorded in the leaves of Cappelle, where the MDA values were consistently higher than 3 nmol/g fresh weight in PEG treatments of 15% or above (Fig. 5). In drought-tolerant plants (Sakha, Ae225, Ae550) there was also an increase in the MDA level in all cases, but the magnitude and rate of this increase and the extent of recovery all differed considerably from those characteristic of plants less successful in counteracting drought stress (Fig. 5).

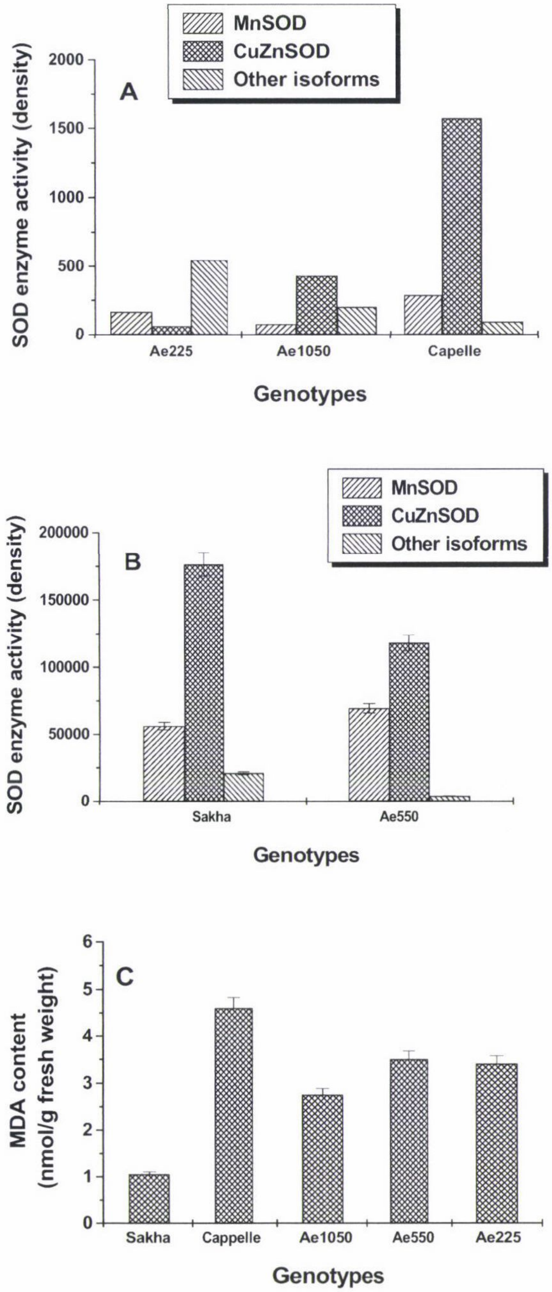

Fig. 3. SOD isoenzyme activity in *Aegilops* (A) and wheat (B) genotypes and MDA content (C) (nmol/g fresh weight) during recovery, 7 days after supplying the plants with nutrient solution without PEG (–0.03 MPa)

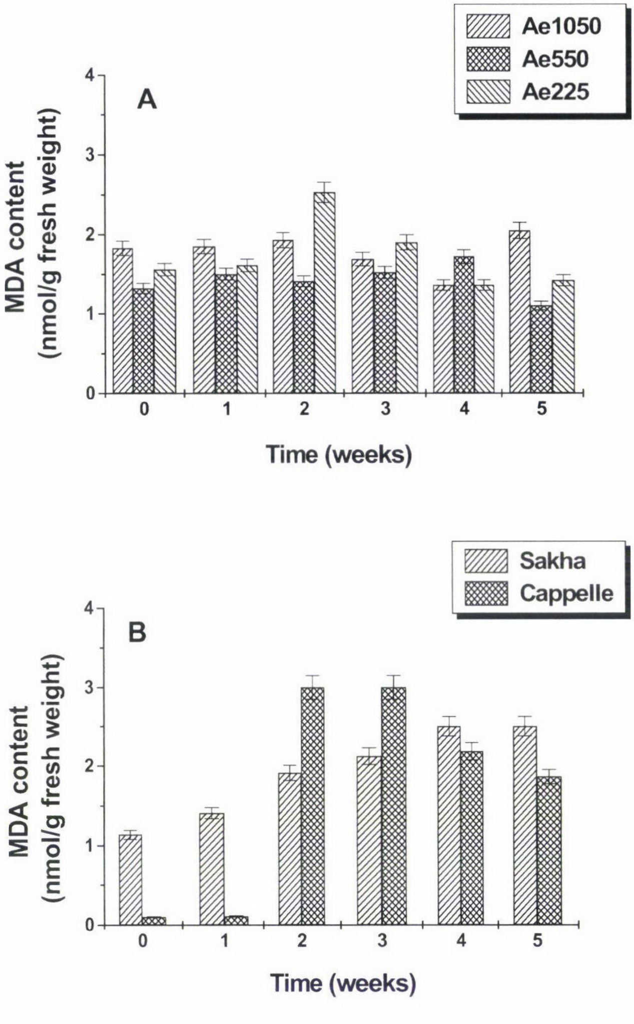

Fig. 4. MDA content in control leaves (nmol/g fresh weight) as a function of the growth period (weeks). *Aegilops biuncialis* genotypes (A) and *T. aestivum* varieties (B) were grown under control conditions

During the recovery period after stress treatment, when the parameters shifted back towards the control values, the changes in the MDA level were less clear. In tolerant plants a uniform change in the MDA level was observed during the treatments, though this varied in the three varieties and accessions (Fig. 5). The MDA content in the leaves of Sakha reached a maximum (3 nmol/g fresh weight) at a PEG concentration of 15%, declining again at higher PEG contents and remaining at this low level during recovery (Figs. 3C, 5). In Ae225 a gradual increase was observed up to a maximum in the 21% PEG treatment (4.1 nmol/g fresh weight), followed by a decrease during recovery (Figs. 3C, 5). In the leaves of Ae550 plants an ever greater increase was recorded from the start of treatment until the control state was regained (Fig. 5). This was extremely unusual for a drought-tolerant plant, and suggested a quite different tolerance strategy.

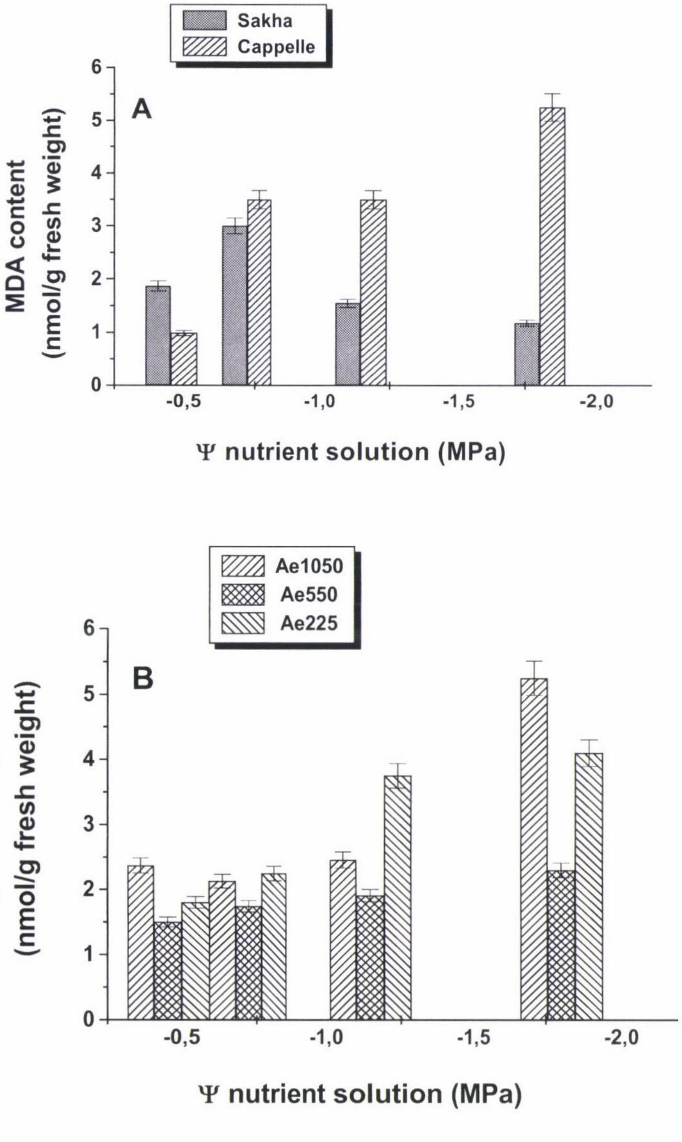

Fig. 5. Change in the MDA content (nmol/g fresh weight) of PEG-stressed leaves of wheat varieties (A) and *Aegilops biuncialis* genotypes (B) as a function of the water potential of the nutrient solution (MPa). Measurements were made at the end of the 7-day treatment periods (water potential of the nutrient solution was as follows: 0–7 days: –0.46 MPa; 7–14 days: –0.72 MPa; 14–21 days: –1.15 MPa; 21–28 days: –1.80 MPa)

In plants where a high MDA level was observed during the treatments (the drought-sensitive Ae1050 and Cappelle), there was again a reduction in the MDA concentration during the recovery period, in accordance with the general tendency (Fig. 3C).

The actual efficiency of PSII photochemistry ($\Delta F/F_m$'), which is proportional to the electron transport rate at 200 μmol m^{-2} s^{-1} growth light intensity, was assessed on detached leaves pre-treated with 30 mM glyceraldehyde to block the regeneration of ribulose 1,5-bisphosphate from triose phosphate and thus effectively inhibit CO_2 fixation. As a result of glyceraldehyde pre-treatment, there was a substantial decrease (of 40–60%) in the $\Delta F/F_m$' parameter in control plants in all stages and in all the genotypes investigated (Fig. 6). The remaining part of $\Delta F/F_m$' reflects the intensity of photosynthetic electron flow to sinks other than CO_2 fixation.

In plants subjected to drought stress, $\Delta F/F_m$' remained higher than in their control counterparts, although to differing extents. The difference between PEG-treated and control plants could be attributed to the induction of alternative electron transport processes such as the Mehler reaction, which can be considered as a protective mechanism against the overreduction of electron transport components. The induction of these alternative electron transport processes could be observed in all the genotypes, but to differing extents and at different water potentials in the nutrient solution. The intensity of alternative electron transport processes reached a maximum when drought stress was moderate (–0.7 MPa) in the three *Ae. biuncialis* genotypes (5–10% inhibition of $\Delta F/F_m$' with glyceraldehyde) and in the *T. aestivum* variety Sakha (15% inhibition of $\Delta F/F_m$'). It has to be emphasized that at –0.7 MPa of water potential stomatal conductance was already decreased substantially in all genotypes (Molnár et al., 2004), which explains why these processes were accelerated at this relatively early stage of drought stress. Nevertheless, in the case of Cappelle, the induction of alternative electron transport processes could also be detected, though to a lesser extent, as 20% of $\Delta F/F_m$' was inhibited by glyceraldehyde even under severe drought stress (water potential of –1.8 MPa). After a 7-day recovery phase, the relaxation of the alternative electron transport processes could be observed in Ae1050, while in all the other genotypes $\Delta F/F_m$' remained at the level observed during severe drought stress, or increased even further.

The following order of drought stress tolerance was determined on the basis of the results: Sakha, Ae550 > Ae225 > Ae1050 >> Cappelle. The SOD enzyme activity range and the MDA content were very similar in the Sakha and Ae550 plants and differed significantly from those of the other genotypes. The varieties and accessions found to be tolerant of drought (the three *Aegilops* genotypes and Sakha) showed very different responses, both in terms of lipid peroxidation and as regards the inducibility of alternative electron transport processes; in the first case Ae550 and in the second Ae1050 exhibited behaviour different to that observed for the other varieties and accessions. The results definitely indicate that even in varieties with a similar level of stress tolerance efficient protection may be achieved using different mechanisms related to the different genetic background. Experiments will be required on a larger collection of varieties if the various tolerance strategies are to be more accurately described.

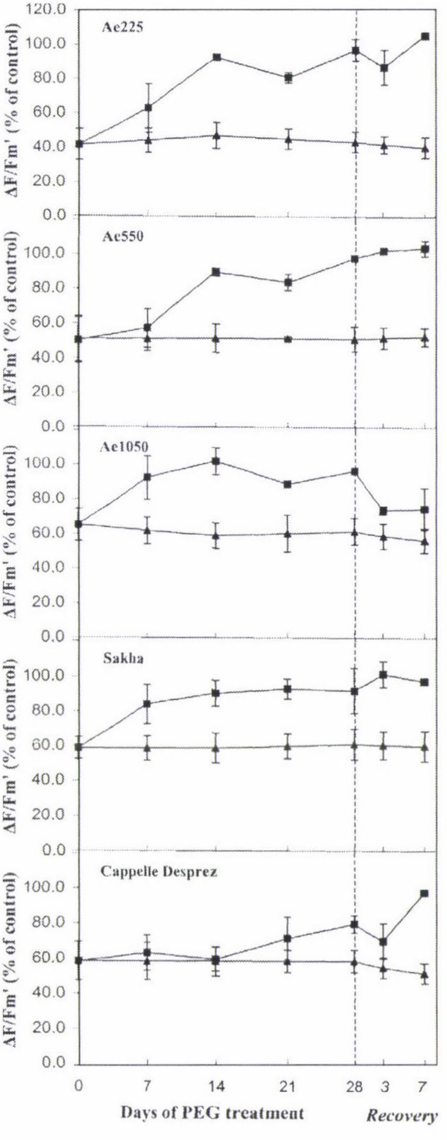

Fig. 6. Effective quantum efficiency of photosystem II ($\Delta F/F_m'$) of control (full triangles) and PEG-treated (full squares) *Ae. biuncialis* genotypes and *T. aestivum* varieties. Measurements were made at the end of the 7-day treatment periods (water potential of the nutrient solution was as follows: 0–7 days: –0.46 MPa; 7–14 days: –0.72 MPa; 14–21 days: –1.15 MPa; 21–28 days: –1.80 MPa) and 3 and 7 days after supplying the plants with nutrient solution without PEG (–0.03 MPa). Detached leaves were preincubated in 30 mM glyceraldehyde for 2 h to block the Calvin-Benson cycle. Data are presented as a percentage of the control values measured on intact leaves without the inhibition of CO_2 fixation

Acknowledgements

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References

Beauchamp, C., Fridovich, I (1971): Superoxide dismutase. Improved assays and an assay applicable to acrylamide gel. *Anal. Biochem.*, **44**, 276–287.

Borsania, O., Díaza, P., Agiusa, M. F., Valpuestac, V., Monza, J. (2001): Water stress generates an oxidative stress through the induction of a specific Cu/Zn superoxide dismutase in *Lotus corniculatus* leaves. *Plant Sci.*, **161**, 757–763.

Fridovich, I. (1986): Superoxide dismutases. *Adv. Enzymol.*, **58**, 61–97.

Harris, J. I., Auffret, D., Northrop, F. D., Walker, J. E. (1980): Structural comparisons of superoxide dismutases. *Eur. J. Biochem.*, **106**, 297–303.

Heath, R., Packer, L. (1968): Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch. Biochem. Biophys.*, **125**, 189–198.

Molnár, I., Gáspár, L., Sárvári, É., Dulai, S., Hoffmann, B., Molnár-Láng, M., Galiba, G. (2004): Physiological and morphological responses to water stress in *Aegilops biuncialis* and *Triticum aestivum* genotypes with differing tolerance to drought. *Funct. Plant Biol.*, **31**, 1149–1159.

Nagy, Z., Galiba, G. (1995): Drought and salt tolerance are not necessarily linked: a study on wheat varieties differing in drought resistance under consecutive water and salinity stresses. *J. Plant Physiol.*, **145**, 168–174.

Selote, D. S., Bharti, S., Khanna-Chopra, R. (2004): Drought acclimation reduces O_2*-accumulation and lipid peroxidation in wheat seedlings. *Biochem. Biophys. Res. Commun.*, **314**, 724–729.

Tischner, T., Kőszegi, B., Veisz, O. (1997): Climatic programmes used in the Martonvásár phytotron most frequently in recent years. *Acta Agron. Hung.*, **45**, 85–104.

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EFFECT OF OLIVE JIFT AND SUBLETHAL GLYPHOSATE APPLICATIONS ON FABA BEANS (*Vicia faba*)

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Greenhouse experiments were conducted to determine the effects of mixing olive mill by-product (jift) into the soil or of applying sublethal rates of glyphosate at various times on faba beans. The efficacy of these applications to control broomrape is currently being evaluated in Jordan. In one experiment, faba beans were grown in rooting mixtures that contained jift in calculated percentages of 0, 14.3, 16.6, 20, 25, 33.3 and 50% (v/v). In the second experiment, the foliar parts of faba bean plants were sprayed with glyphosate at a rate of 40 g ai/ha applied at either 1, 2, 3, 4, 5, 6, 7 or 8 weeks after crop emergence. The results indicated that mixing jift with the soil did not affect most variables measured above or below ground on current or subsequent faba bean crops. Slight reductions in plant height and dry weight were recorded sporadically. A decrease in the pH of the rooting mixture was observed as the percentage of jift increased. Electrical conductivity values also increased as the percentage of jift in the mixture increased. Sublethal glyphosate applications did not cause any effect on faba bean growth and yield. The results of both experiments indicate that these two methods, if they prove to be effective for broomrape control, have no adverse effects on the growth and yield of faba beans.

Key words: broomrape, glyphosate, olive jift, *Orobanche* spp., parasitic weeds, *Vicia faba*, weed control

Introduction

Faba beans (*Vicia faba* L.), a valuable food legume crop in Jordan and many other Mediterranean countries, occupy nearly 3.6×10^6 ha worldwide (Karamanos et al., 1994). The yield of faba beans may become unstable as the result of weed invasion, diseases and insect attacks (Hawtin and Hebbelthwaite, 1983). Broomrapes (*Orobanche* spp.) are the main weeds that restrict the growth of faba beans in tropical and subtropical climates (Jain and Foy, 1989). These non-photosynthesizing root-parasitic plants obtain their carbon, water and

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nutrients from their host through haustorial connections (Nandula et al., 1996). Broomrapes cause damage to host crops by reducing yield, and sometimes even destroy the marketability of the product. Losses in faba beans yields are between 5 to 100% in Egypt, Malta, Morocco, Spain and Turkey due to broomrape infestations (Sauerborn and Saxena, 1987).

Broomrape control in cropping areas is extremely difficult. The application of sublethal doses of glyphosate ([N-phosphonomethyl]glycine) to the host is a common and effective practice (Foy et al., 1989). This chemical is a non-selective herbicide that is readily translocated to the meristematic regions of plants. Glyphosate applied post emergence at about 60 g/ha efficiently controls broomrape in many crops (Jacobson and Levy, 1987). This indirect application is effective whenever it is applied at the proper time and at the proper rate (Lolas, 1986). High rates can effectively control the parasite but will severely injure the crop, whereas broomrape plants will not be affected by low rates of glyphosate. The time of application is also very crucial, since any delay in the treatment may mean that the parasite is no longer sensitive (Basler, 1980).

Jift, a solid by-product of olive oil milling, was introduced recently as a soil amendment for broomrape control. Jift, which consist of 93.52% organic matter, 3.5% ash, 2.41% N and 0.115% P, has a pH of 5.2. Mixing soil and jift at different rates reduced broomrape infections significantly in faba beans, peas (*Pisum sativum*) and tomatoes (*Lycopersicon esculentum*) (Ghosheh et al., 1999). Previously, olive jift was reported to be suppressive to root-knot nematodes (*Meloidogyne* spp.) when incorporated in the soil (Rodriguez-Kabana et al., 1995). Reports regarding the influence of jift on crop growth are uncertain. More than 50 phenols have been identified in olive mill water, together with alcohol, aldehydes and other small organic molecules. These by-products also contain fibres, lignin, tannins and fatty acids that inhibit plant growth (Pages et al., 1985). Propionic and other low molecular weight monocarboxylic acids, which are toxic to many plant species, are also present (Pera and Calvet, 1989). Hameed and Foy (2000) found that olive oil extraction by-products had allelopathic activities, as the growth of some broad-leaved and grass weeds were either inhibited or suppressed.

Since the alternatives available to achieve satisfactory broomrape control levels are limited, the use of sublethal doses of glyphosate or jift as a soil amendment could be vital to overcome problems associated with these parasitic infestations. The time of application has not been properly identified for Jordanian crops. Glyphosate applied to a common vetch experimental field at Maru Research Station in 1997 did not eliminate the broomrape plants that infested the field. Therefore, the objectives of the present work were: a) to detect the influence of mixing jift with soil at variable percentages on current and subsequent faba bean plants; and b) to determine the effect of time of application of a sublethal glyphosate dose on the growth and yield of faba beans in Jordan.

Materials and methods

Influence of jift on faba bean plants

Greenhouse experiments were conducted at the Jordan University of Science and Technology in the 1998 and 1999 seasons. The experimental unit was a single 3-kg plastic pot planted with one faba bean plant. Seeds of the faba bean cv. Major were planted on December 29, 1997 and on November 17, 1998. Treatments that consisted of rooting mixtures with calculated percentages of 0, 14.3, 16.6, 20, 25, 33.3 and 50% jift/soil (v/v) were replicated three times in a randomized complete block design (RCB). These percentages were selected because this is the range within which jift will be used to reduce broomrape infection (Ghosheh et al., 1999). Because the experiment included destructive measurements, nine replicates per treatment were initially established in the first-run experiments. Second-run experiments were initiated to detect any adverse effects from mixing jift with the soil on subsequent crops. The rooting media from all first-run experiments were allowed to dry and new faba beans plants were planted. Two second-run experiments were established on November 17, 1998 (323 days after the first planting) and on May 5, 1999 (173 days after the first planting). The experimental units in these second-run experiments were established as described above and were arranged in a RCB design with three replications.

Plant response to jift mixtures was monitored weekly in both seasons by recording plant height (cm), the number of leaves per plant and the number of branches per plant. Destructive measurements of total leaf area (cm^2) were recorded 4 and 8 weeks after crop emergence (WAE). Fresh and dry (70°C for 24 h) above- and belowground biomass weights were recorded at 4, 8 and 13 WAE (maturity). The number of active nodules on the faba bean plants was determined by counting large pink nodules as described by Roughley et al. (1983). At the onset of the reproductive stage, the number of flowers, the number of pods and pod dry weight were recorded. In the second-run experiments, only measurements of aboveground dry weight were recorded. In order to detect any alteration in soil pH or electrical conductivity (EC), samples from each treatment were taken at 4 and 8 WAE, at the harvest of the first-run experiments, and at the termination of the second-run experiments in each season. Soil pH was determined as described by McLean (1982), while EC values were determined as described by Roades (1982).

The data were analysed according to the regression procedures outlined by Steel and Torrie (1980). The pH and EC data were subjected to ANOVA as outlined for a two-factor randomized complete block design. Mean separation was performed using Fisher's LSD test at $P = 0.05$. All proportional and counted data were arcsin or square root transformed, respectively, to linearize data, as described by Little and Hill (1978). Because of the significant season × treatment interactions, the data for each year were analysed separately.

Influence of sublethal glyphosate application time on the growth and yield of faba beans

Greenhouse experiments were established in the same location and seasons and with the same experimental units as described in Experiment 1. Seeds of faba bean cv. Major were planted on November 17, 1998 and February 5, 1999. A sublethal glyphosate dose of 40 g ai/ha was applied at 1, 2, 3, 4, 5, 6, 7 or 8 WAE using a lever-operated knapsack sprayer (LOK). An untreated check was also included in the experiment. All treatments were arranged in a RCBD with three replications.

The plant response to sublethal glyphosate doses was monitored by recording plant height (cm), visual plant injury ratings on a scale from zero (no injury) to 100% (complete injury), the number of leaves/plant and the number of branches/plant. These measurements were recorded on the untreated check and on experimental units that had received herbicide treatments in previous weeks. At the onset of the reproductive stage, the number of flowers/plant and pods/plant were recorded. Fresh and oven dry (70°C for 24 h) above- and belowground weights were recorded at maturity.

Data collected for the variables recorded weekly were subjected to separate ANOVA that included glyphosate-treated experimental units and untreated checks. For example, the analysis of the plant height recorded 6 weeks after emergence included the heights of two untreated checks and the heights of plants treated with glyphosate at 1, 2, 3, 4 and 5 weeks after faba bean emergence, and so on. All proportional and counted data were arcsin or square root transformed, respectively, to linearize data as described by Little and Hill (1978). Because of the significant season × treatment interactions, the data for each year were analysed separately.

Results and discussion

Influence of jift on faba beans plant

In 1998, an increase in the jift percentage in the rooting mixture affected the height of faba beans adversely when measured 1 or 2 WAE (Fig. 1 and Fig. 2, respectively). Later, however, the presence of jift in the mixture had no effect on plant height. A negative relationship was also observed in the second-run experiment of that season for all measurements recorded 5 or more WAE (Fig. 3 and Fig. 4). These results could be attributed to the fact that olive oil extraction produces a solid by-product that contains fibres, lignins and tannins, which makes it a candidate for allelopathic activity (Hameed and Foy, 2000). Allelopathy, the adverse effects on the growth of plants or microorganisms caused by the action of chemicals produced by other living or decaying plants (Ahrens, 1994), could have played a role in reducing the height of faba beans on these occasions. However, the inconsistency and the slight magnitude of the height reduction do not raise serious concerns regarding potential adverse effects on faba beans from adding jift to the soil.

Mixing jift with the soil at the percentages specified in these experiments did not affect leaf number/plant or area, branches/plant or flowers/plant. These results indicate that jift in the rooting mixture did not adversely affect the growth or floral development of faba beans, and that its use as a soil amendment to control broomrape is not deleterious.

The presence of jift in the mixture did not affect the number of pods in any of the experiments, while the pod dry weight only increased with increasing jift percentage in the 1998 first-run experiment (Fig. 5). This increase in dry matter production could be attributed to the ability of jift to enrich the soil with minerals and hence to improve plant growth, as previously described by Hamdi (1993), who related soil nutrient enrichment with the successful use of olive mill waste water to enrich agricultural lands.

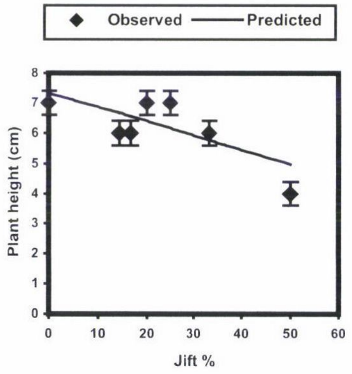

Fig. 1. Influence of different percentages of jift in rooting mixtures on faba bean height (cm) measured 1 week after emergence in the 1998 season. Regression equation was Y = 7.33 – 0.047 X (r = –0.438), bars indicate Standard Error

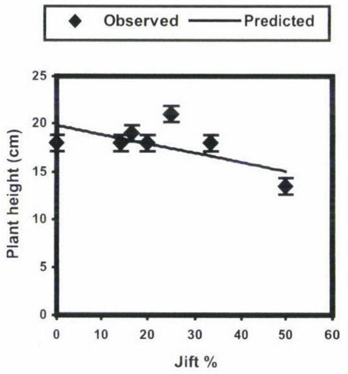

Fig. 2. Influence of different percentages of jift in rooting mixtures on faba bean height (cm) measured 2 weeks after emergence in the 1998 season. Regression equation was Y = 19.81 – 0.095 X (r = –0.379), bars indicate Standard Error

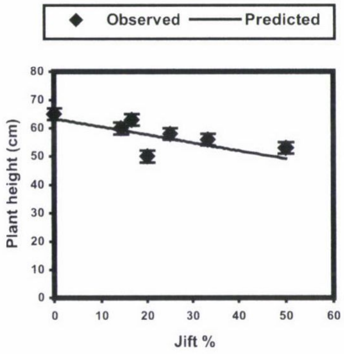

Fig. 3. Influence of different percentages of jift in rooting mixtures on faba bean height (cm) measured 5 weeks after emergence in the 1998 second-run experiment. Regression equation was $Y = 63.38 - 0.284\ X$ ($r = -0.489$), bars indicate Standard Error

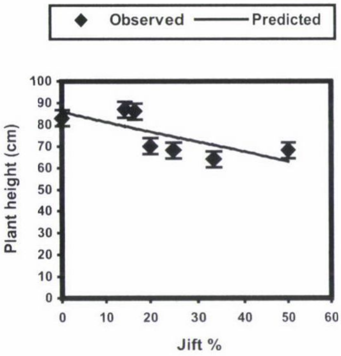

Fig. 4. Influence of different percentages of jift in rooting mixtures on faba bean height (cm) measured 13 weeks after emergence in the 1998 second-run experiment. Regression equation was $Y = 85.84 - 0.459\ X$ ($r = -0.510$), bars indicate Standard Error

Aboveground dry weights were not affected by adding jift to the rooting media when destructive measurements were recorded at 4, 8 or 13 WAE in 1998 or at the termination of the second-run experiment of that season. In 1999, there was no significant effect on aboveground dry weight after 4 or 8 WAE. However, measurements recorded 13 WAE indicated that aboveground dry weight was reduced by increasing the percentage of jift in the mixture (Fig. 6). This reduction in stem and leaf dry weights with an increase in the percentage of jift was attributed to the presence of allelopathic compounds in jift, as described by Pages et al. (1985).

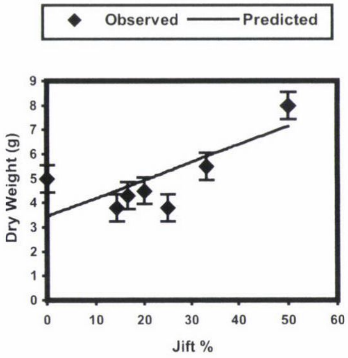

Fig. 5. Influence of different percentages of jift in rooting mixtures on faba bean pod dry weight (g) measured 91 days after emergence in the 1998 season. Regression equation was $Y = 3.45 + 0.074\ X$ ($r = 0.589$), bars indicate Standard Error

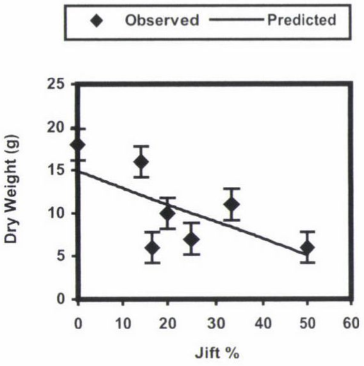

Fig. 6. Influence of different percentages of jift in rooting mixtures on the dry weight (g) of faba bean stems and leaves measured 91 days after emergence in the 1999 experiment. Regression equation was $Y = 14.92 + 0.0196\ X$ ($r = -0.511$), bars indicate Standard Error

Mixing jift with the soil adversely affected the nodule number per plant in 1998 when measured 4 WAE (Fig. 7), but not at 8 WAE. In 1999, there was no significant effect of jift on the nodule number at either 4 or 8 WAE. Apparently, nodule development was delayed but not prohibited in the presence of jift in the rooting zone in the 1998 experiment, and this delay could be related to possible allelopathic effects. One observation that is worth mentioning is that in 1999 the quantity of allelopathic compounds may have been reduced by applying irrigation water prior to planting the faba bean seeds. The water might have reduced the allelopathic compounds in a manner similar to that described by Pages et al. (1985).

Fresh and dry root weights were not affected by the presence of jift in the soil when measured 4 or 8 WAE. The roots were not extracted after harvest because the soil was kept undisturbed to establish second-run experiments. Soil pH, which largely controls the plant nutrient availability and microbial reaction in soils (Foth, 1984), decreased as the percentage of jift in the mixture increased in both seasons. On the other hand, the pH value only increased with duration in 1998 (Table 1, 7.68 vs. 7.97). Similarly, the EC of the rooting mixtures increased in the first season as the jift percentage increased, but was not affected by the duration after mixing. In 1999, EC values increased with increasing jift percentage or duration after mixing (Table 1). The reduction in soil pH due to adding jift is considered advantageous in soils of a basic nature. Pera and Calvet (1989) found that olive extracts have a pH range of 7.0 to 7.2. Therefore, mixing jift with soil to control broomrape will probably reduce problems associated with basic soils. High EC values are advantageous to a certain extent, since this is indicative of a high concentration of total solutes in the soil.

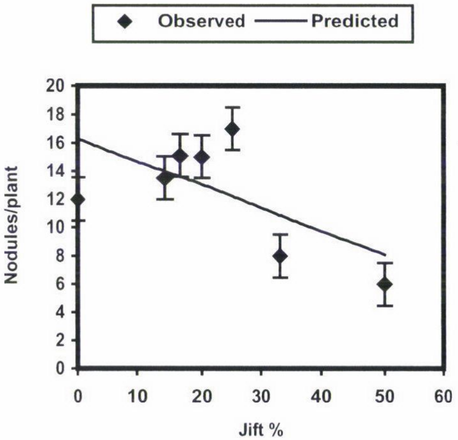

Fig. 7. Influence of different percentages of jift in rooting mixtures on faba bean nodule number measured 28 days after emergence in the 1998 season. Regression equation was $Y = 16.32 - 0.164 X$ $(r = -0.460)$, bars indicate Standard Error

Table 1
Effect of jift percentage in rooting mixture and duration after mixing on the pH and electrical conductivity values recorded in greenhouse experiments

Treatments	Mixture pH		Electrical conductivity μς/cm[1]	
	1998	1999	1998	1999
Jift (%)				
0.0	8.01 a[2]	8.05 a	440.3 b	384.1 b
14.3	8.00 a	8.04 a	615.0 a	558.8 a
16.6	7.89 a	8.030 ab	684.0 a	657.4 a
20.0	7.79 ab	8.030 ab	680.7 a	627.6 a
25.0	7.83 ab	7.92 bc	606.0 a	593.3 a
33.3	7.64 bc	7.92 bc	675.8 a	657.6 a
50.0	7.40 c	7.85 c	717.5 a	625.8 a
Duration after mixing (weeks)				
4	7.68 b	7.97 a	671.1 a	568.4 b
8	7.72 b	7.94 a	574.4a	564.6 b
13	7.80 ab	8.00 a	654.5 a	534.6 b
34 or 26[3]	7.97 a	7.97 a	625.7 a	679.1 a

[1]μς/cm : Microsiemens per centimetre; [2]Means within a column for either jift % or duration followed by the same letter were not different according to Fisher's LSD test (P≤0.05). [3]The second-run experiment was initiated and terminated earlier in 1999 than in 1998.

Influence of sublethal glyphosate application time on the growth and yield of faba beans

Glyphosate applications at various times did not affect the height of faba beans and did not cause any visual injury throughout the two seasons. The variables leaves/plant, branches/plant, flowers/plant, number and dry weight of pods, aboveground dry weight, and fresh and dry root weights were not affected by glyphosate applications in either season (data not presented). These results agree with those of Sauerborn et al. (1989), who stated that glyphosate phytotoxicity is reduced at rates of 60 g ai/ha and below. This indicates that faba bean plants can tolerate a sublethal glyphosate dose of 40 g ai/ha when applied at any growth stage to achieve the best control of broomrape.

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References

Ahrens, W. H. (1994): *Herbicide Handbook* (7th Ed.). Weed Science Society of America. p. 313.
Basler, F. (1980): Lentil weed research in ICARDA. *Lens,* **6**, 58–60.
Foth, H. P. (1984): *Fundamentals of Soil Science.* Wiley and Sons, Inc., USA. pp. 197–200.

Foy, C. L., Jain, R., Jacobson, R. (1989): Recent approaches for chemical control of broomrapes (*Orobanche* spp.). *Rev. Weed Sci.*, **4,** 123–152.

Ghosheh, H. Z., Hameed, K. M., Turk, M. A., Al-Jamali, A. F. (1999): Olive (*Olea europea*) jift suppresses broomrape (*Orobanche* spp.) infection in faba beans, peas, and tomatoes. *Weed Technology,* **13,** 457–460.

Hamdi, M. (1993): Future prospects and constraints of olive mill wastewater use and treatment: A review. *Bioprocess Engineering,* **8,** 209–214.

Hameed, K. M., Foy, C. L. (2000): Potential utilization of olive (*Olea europea*) pomace (Jift) from olive oil mills as a bioherbicide. *Weed Science Society of America 40th Annual Meeting (WSSA), Toronto, Canada*, **40,** 115.

Hawtin, G. C., Hebblethwaite, P. D. (1983): Background and history of faba beans production. pp. 3–22. In: Hebblethwaite, P. D. (ed.), *The Faba Beans (Vicia faba), A basis for Improvement.* Butterworths, London.

Jacobsohn, R., Levy, D. (1987): Glyphosate for *Orobanche* control in various crops, problems and promises. pp. 172–178. In: Pieterse, A. H. (ed.), *Biology and Management of Orobanche.* Proceedings of the Third International Workshop on *Orobanche* and Related Research. Royal Tropical Institute, Amsterdam, The Netherlands.

Jain, R., Foy, C. L. (1989): Broomrapes (*Orobanche* spp.) a potential threat to U.S. broad leaf crops. *Weed Technology,* **3**, 608–614.

Karamanos, A. J., Papadopulos, G., Avogulas, C., Papastylianon, P. (1994): Chemical composition of seeds of 11 field-grown faba bean cultivars in two cultivation periods. *FABIS*, **34/35**, 39–47.

Little, T. M., Hills, J. F. (1978): *Agriculture Experimentation: Design and Analysis.* Wiley and Sons Inc., USA, ISBN: 0-471-02352-3.

Lolas, P. C. (1986): Control of broomrape (*Orobanche ramosa*) in tobacco (*Nicotiana tabacum*). *Weed Sci.,* **34**, 427–430.

McLean, E. O. (1982): Soil pH and lime requirement. pp. 59–69. In: Page, A. L., Miller, R. H., Keeney, D. R. (eds.), *Methods of Soil Analysis. Part II (2nd Ed.)* American Society of Agronomy-Soil Science Society of America, Madison, Wisconsin, USA.

Nandula, V. K., Foy, C. L., Westwood, J. H. (1996): Environmental influence on germination of *Orobanche. Proc. 6th Parasitic Weed Symp.,* Cordoba, Spain. pp. 409–416.

Pages, M., Estaun, V., Calvet, C. (1985): Physical and chemical properties of olive marc compost. *Acta Horticulturae,* **172**, 271–276.

Pera, J., Calvet, C. (1989): Suppression of *Fusarium* wilt of carnation in a composted pine bark and composted olive pumice. *Plant Disease*, **73,** 699–700.

Roades, J. D. (1982): Soluble salts. pp. 69–78. In: Pages, A. L., Miller, R. H., Keeney, D. R. (eds.), *Methods of Soil Analysis. Part II (2nd Ed.)*, American Society of Agronomy-Soil Science Society of America, Madison, Wisconsin, USA.

Rodriguez-Kabana, R., Eastaun, V., Pinochet, J., Marfa, O. (1995): Mixtures of olive pomace with different nitrogen sources for the control of *Meloidogyn* spp. on tomato. *Journal of Nematology,* **27 (4S),** 575–584.

Roughley, R. J., Spent, J. I., Day, J. M. (1983): Nitrogen fixation. pp. 233–256. In: Hebblethwaite, P. D. (ed.), *The Faba Beans (Vicia faba), A Basis for Improvement.* Butterworths, London.

Sauerborn, J., Saxena, M. C. (1987): Effect of soil solarization on *Orobanche* spp. infestation and lentils. pp. 733–744. In: Weber, H. C., Forstrenter, W. (eds.), *Parasitic Flowering Plants, Proceeding of the 4th International Symposium of Flowering Parasitic Plants.* Marburg, Germany.

Sauerborn, J., Saxena, M. C., Meyer, A. (1989): Broomrape control in faba beans (*Vicia faba* L.) with glyphosate and imazaquin. *Weed Research,* **29,** 103–111.

Steel, R. G. D., Torrie, J. H. (1980): *Principles and Procedures of Statistics. A Biometric Approach.* 2nd ed. McGraw-Hill, New York, USA.

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MAIZE VARIETIES IN EASTERN CENTRAL EUROPE IN THE FIRST DECADES OF THE 20TH CENTURY

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Since a variety registration system was introduced in Hungary in 1914, all the necessary information about varieties improved by professional breeders is made public. However, little is known about the origin of varieties bred by local farmers for their own purposes in Eastern Central Europe. The catalogue of the First National Maize Exhibition, held in Budapest in 1914, provides a unique opportunity to investigate the genetic background of the maize varieties of the time. It seems likely that the diversity of this genetic background was preserved until the beginning of hybrid maize breeding.

The flint varieties of the time proved to be the most variable (Caribbean, Andean, Paduan and Northern flints). Among the Corn Belt Dents, Leaming, Queen of the Prairie, Reid Yellow Dent, Iowa Goldmine and Northwestern Dent were the most frequent varieties, while Tuxpan, Gourdseed, Shoepeg, Hickory King and Southern Prolific were the most frequent of the Southern Dent varieties. In many cases the varieties introduced into Eastern Central Europe mixed and crossed spontaneously. In addition to professional breeders, many farmers also used the available varieties as components in crosses, in order to develop new varieties. The most popular were dent × flint crosses, using roughly equal proportions of Old Hungarian Yellow Flints of the Caribbean type and early hard-grained flints of the Andean type. Flint × flint crosses were also popular, partly due to the use of maize for human consumption, and partly to the great genetic variability exhibited by flint varieties. Locally developed maize varieties, which have a background quite different from those developed in the North American Corn Belt, could, after suitable breeding, enrich the available sources of heterosis. Further research will be required to determine which of them are the most valuable.

Key words: maize, breeding, diversity, genetic resources

Introduction

The world fairs organised between 1880 and 1910 included exhibitions of plant varieties and animal breeds. Hungarian open-pollinated maize varieties were shown with great success at these exhibitions (Legkorábbi Székely, Lapusnyaki, etc.). During this period maize was grown on 2.5–3 million hectares in Hungary, with yields of 1.5–2.2 t/ha, making Hungary the third ranking maize-producing country in the world after the United States and Argentina (Surányi, 1914). In Eastern Central Europe maize was grown for both human (porridge) and animal consumption. It was a period of rapid economic development, and great interest was shown in the introduction and spread of new tools, methods and techniques for maize production and in the introduction of new maize varieties capable of producing stable, high yields.

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At the annual meeting of the National Hungarian Farming Association (OMGE) in 1911 a resolution was passed to organise an independent maize exhibition, which was finally held in 1914. Regional maize shows were held earlier (Temesvár 1910, Torontál County, 1913) and later (Debrecen 1924, Székesfehérvár 1940), but the national exhibition held in Budapest in 1914 was not repeated. A catalogue was published detailing the varieties shown at the exhibition, and this serves as a unique source of the names and origins of the contemporary maize varieties and of the extent to which they were grown, aiding investigations into their genetic backgrounds.

Many papers were published on the maize varieties grown, bred and introduced in Eastern Central Europe (Agr. Z., 1855; Szentkirályi, 1877; 1881; Cserháti, 1895; Csíktapolcai-Lázár, 1899; Lubinszky, 1901; 1903; Barna, 1904; Röszler, 1907; Grábner, 1908; 1903; 1916; 1917; 1922; 1929; Zathureczky, 1910; Butujás, 1912; Péterfy, 1912a; b; c; d; 1913a; b; c; Fleischmann, 1913a; b; Tímár, 1913; Kenyeres, 1913; Baross, 1916; Kárász, 1921; Falb, 1923; Dudás, 1925; Pap, 1929; Villax and Surányi, 1932; Berzsenyi-Janosits, 1951; Taróczy, 1952; 1954; Jánossy et al., 1957; Brandolini, 1968; 1971; Pavlicic, 1971; Trifunovic, 1978; Marton and Németh, 2003; Hadi et al., 2003a; b; c; 2004; Hadi, 2004; 2005a; b; etc.). It is now known that the European Flint, Ruma and Mindszentpuszta sources of heterosis had an origin different from that of the North American Corn Belt varieties and developed independently in Europe for a long period. Their excellent combining ability, based on high yield potential, allowed them to be distinguished from the heterosis sources widespread in the Corn Belt (Reid Yellow Dent, Lancaster) and to be handled separately. In recent years data published by Radovic and Jelovác have suggested that other valuable heterosis sources arose, apart from those listed above.

The 1914 General Meeting of the OMGE also introduced a plant registration system to guarantee the origin and economic value of improved, progeny-tested varieties. However, it did not regulate the sales of seed from maize varieties bred by local farmers. The maize variety and race composition registered and collected for gene bank storage by Jánossy et al. (1957), Leng et al. (1962) and Pavlicic (1971) prior to the introduction of hybrid maize arose jointly from the seed of landraces developed from improved, registered varieties and seed from varieties of unknown origin, bred by local farmers. The catalogue of the First National Maize Exhibition held in Budapest in 1914 provides important information on the origin and genetic background of locally bred varieties, which could be used both for identifying existing sources of heterosis and for creating new ones.

Materials and methods

Individuals, associations and institutions entered one or more samples (varieties) or collections in the maize exhibition. In general, samples consisted of five plants (ear + stalk), but in some cases the samples were 10 ears, or 25 kg of shelled kernels. Each sample was required to have a distinct variety name and origin, to be distinguishable from all other varieties and to be morphologically stable. Samples that were abnormally developed, mouldy, or lacked distinct variety character were not accepted. Under the terms of previously elaborated regulations, a 33-man committee met in secret to judge the entries, and awarded a total of 13 gold, 31 silver and 60 bronze medals in 11 categories, and a large number of certificates.

The secretary, Endre Fabricius, and his deputy, Albert Rész, edited and published the documents relating to the exhibition (Fabricius, 1914).

The material in the exhibition was expanded on several occasions. The catalogue only mentions 2 varieties of sweetcorn, 72 Hungarian hard-grained flint, 162 Hungarian soft-grained flint, 75 Hungarian dent, 150 American dent, 14 American flint and 28 other varieties, amounting to a total of 503 samples (varieties). However, 830 varieties entered by individual exhibitors are listed. In actual fact the catalogue gives information on 1761 varieties, of which 1285 samples (varieties) were adjudicated (Table 1).

In spite of the exhibition rules, many varieties did not have a distinguishing variety name (e.g. White Dent, variety from Jakabszállás, Hungarian Dent, Hungarian × American Dent, Mastodon × Illinois Champion). Nevertheless, the varieties could be identified from the address of the exhibitor, the pedigree and the description of the variety.

In many cases it is impossible to determine whether varieties originally introduced from America, which evolved further in the course of variety maintenance carried out by farmers using seed from the previous year's crop, are landraces (ecotypes) or new farmer-bred varieties. This latter is suggested by the fact that the names of the introduced dent varieties sold by the farmers among themselves were usually written with Hungarian orthography and sometimes bore little relationship to the original. Nevertheless, these were taken to be identical with the original variety. For several varieties it is not clear whether they were dent or flint. All in all, 476 of the exhibited varieties did not meet the requirements. Most of these were of mixed variety, of hybrid origin, or the result of variety maintenance by farmers, and were excluded from the evaluation.

Table 1

Material presented at the First National Maize Exhibition (Budapest, 1914)

No.		Frequency	
		No.	%
	Distribution of varieties according to class of exhibitor		
1	Individual exhibitors	830	47.1
2	Associations	841	47.8
3	Institutions	90	5.1
	Total	*1761*	*100.0*
	Distribution of varieties according to kernel type		
1	Flint varieties	709	40.2
2	Dent varieties	437	24.9
3	Kernel type not identifiable	139	7.9
4	Samples without distinct variety traits	476	27.0
	Total	*1761*	*100.0*

Results

Entries were received from only 32 of the 63 counties found in Hungary prior to 1920 (Table 2). For this reason the Andean hard-grained flint varieties (Legkorábbi Székely, Székely Muskotály, Cinquantino, Pignoletto) grown for human consumption in Transylvania (now Romania) and Upper Hungary (now Slovakia) and the soft-grained flint variety Lapusnyaki, also grown widely in these regions for human consumption, were clearly under-represented in the exhibition material. From the whole of Transylvania only the collection of Kolozsvár University was exhibited, while no exhibits at all were received from Croatia (also then part of Hungary). In contrast, 50% of the total exhibits were entered from five counties (Bácsbodrog, Torontál, Temes, Csanád, Baranya) in the Vajdaság region (now Serbia and Montenegro), which could be considered as the European Corn Belt. These late-maturing, high-yielding dent varieties, which had a high water requirement, were probably over-represented compared with their distribution throughout the country.

Table 2

Samples submitted to the First National Maize Exhibition (Budapest, 1914)

No.	County	Frequency	
		No.	%
1	Bácsbodrog	274	21.3
2	Moson	141	11.0
3	Torontál	137	10.7
4	Csanád	100	7.8
5	Transylvania	100	7.8
6	Fejér	86	6.7
7	Temes	81	6.3
8	Pest	59	4.6
9	Baranya	47	3.7
10	Békés	47	3.7
11	Arad	37	2.9
12	Csongrád	35	2.7
13	Nyitra	32	2.5
14	Veszprém	26	2.0
15	Bars	14	1.1
16	Bihar	10	0.8
17	Somogy	9	0.7
18	Bács	9	0.7
19	Szatmár	8	0.6
20	Komárom	6	0.5
21	Borsod	5	0.4
22	Szabolcs	5	0.4
23	Tolna	4	0.3
24	Pozsony	2	0.2
25	Zala	2	0.2
26	Vas	2	0.2
27	Jász-Nagykun	2	0.2
28	Hajdú	1	0.1
29	Nógrád	1	0.1
30	Zemplén	1	0.1
31	Heves	1	0.1
32	Győr	1	0.1
	Total	*1285*	*100.0*

It is interesting to note that 55% of all the maize varieties with distinct character were of the flint kernel type. Half of the flint varieties (47%) belonged to the Caribbean (Caribbean Flint, Coastal Tropical Flint, Early Caribbean) group (race) of flint varieties that developed in Europe over the course of several hundred years and can be grouped under the name Old Hungarian Yellow Flint (Table 3). The vast majority of these were bred and stabilised by farmers and often differed from each other to a considerable extent. Many of them were marketed by the seed trade (e.g. Muraközi, Drávamenti, Tiszamenti, Szamosmenti, Marosmenti, Bánáti, Bácskai, Fehérvári, Rábaközi, Jászsági, Kispesti, Kisszállási, Mátételki, Galgahévizi, Ecsédi, Hajdúhadházi, Csanádpalotai, etc.). In a few cases a note was also made of the farmers who bred these varieties. The scientific opinion of the time only considered an improved variety to be the result of professional breeding if it was developed using the pedigree method, irrespective of whether the initial stock was a population of a known variety or a planned cross between varieties. The improved varieties from the Old Hungarian Yellow Flint group that are known to have been commercially sold, such as Lészai's or Ugron's variety (Syn: Magyargorbói), Mesterházi's variety (Syn: Nagygeresdi) and the famous Korai Bánáti variety were also referred to and marketed under many other names: Korai Bánsági, Zsombolyai, Legkorábbi Magyar, Saági, Temes-saági, Szentgyörgypusztai, Zathureczky's, János Tímár's, Tallián's, Csekonics's, Algyógyi Sokcsövű (Syn: Gáspár's, Pekri's) Pallagi (Syn: Debreceni), etc.

Table 3
Flint maize varieties (Budapest, 1914)

No.	Name of variety	Frequency	
		No.	%
1	Old Hungarian Yellow Flint	333	47.0
2	Putyi	96	13.5
3	Páduai	65	9.2
4	Cinquantino	59	8.3
5	Alcsúti pignolettó	55	7.8
6	Legkorábbi Székely	33	4.7
7	Florentini	3,1	4.4
8	Lapusnyaki	11	1.6
9	Pennsylvania 8 rows	9	1.3
10	Mauthner's	7	1.0
11	King Philip	5	0.7
12	Canadian Early Yellow	3	0.4
13	Moldvai	2	0.3
	Total	*709*	*100.0*
	According to genetic background		
	Central European soft flint	344	48.5
	Central European hard flint	243	34.3
	Paduan flint	65	9.2
	French flint	31	4.4
	Northern flint	24	3.4
	Romanian flint	2	0.3
	Total	*709*	*100.0*

The Old Hungarian Yellow Flint group is very probably identical with the Derived Flint group designated by Leng et al. (1962), from which the Bosanac, Hrvatska and Timók varieties originated. Most of these varieties were of the soft flint type, with 14 (12–16) kernel rows, thick cobs and long ears. It is possible that a small number of them were flint × flint hybrids. There seems to be little ground for assuming that the whole of the Old Hungarian Yellow Flint group, of Caribbean origin, consisted of hybrids or mixtures of various races.

Varieties related to the Cinquantino (Putyi, Legkorábbi Székely) or Pignoletto (Alcsúti, Béllyei, Zalaszentgróti, Esterházi, Bánkúti Flint) races, of Andean origin, which were also used for human consumption (porridge), made up 34.7% of the flints.

Another contribution to the genetic variability of the Eastern Central European flints was made by varieties from Padua, which were probably related to the Guatemalan conical flints (varieties later bred: Pál Kárász's Prolific Paduan, Mindszentpusztai White, Lovászpatonai White, Manó Szold's Paduan, etc.).

Contrary to expectations, the ratio of confirmed Northern Flints among the flint varieties was only 3.4% (Mauthner 12-week = North Dakota Flint, Pennsylvania 8-row, King Philip, Canadian Yellow Flint). It seems probable that the Northern Flints made little contribution to the development of the genetic background of maize (either flint or dent) in Eastern Central Europe.

Both Corn Belt Dents and Southern Dents are to be found among the dent varieties (Table 4). The Southern Dents included Livingstone Early Golden, of the Gourdseed type, Szalontai Dent and Mastodon, of the Shoepeg type, White Dents (Mammoth, Bristol) of the Tuxpan type, including the improved varieties Gyérei-Dudás's White Dent, and two varieties of the Hickory King type, Fehérvári Fillér and New Golden Giant. The Southern Prolific variety was also present. Altogether Southern Dents made up 21.7% of the dents. The ratio of dents that were certainly bred in Hungary (Garabosi Golden Dent, Bánkúti Late Dent, Gyérei-Dudás's White Dent, Szalontai Dent, Szegényember Kukoricája, Fehérvári Fillér and Red Dent) amounted to 38.1%. Later, several variants of Red Dent became widespread: Szőregi, Ecsedi, Tordasi, Kötegyáni Red Dents. These were related to the varieties Northwestern Dent or Bloody Butcher. The variety known as Szegényember kukoricája (Poor Man's Corn) was not identical with the American variety of the same name, also differing from it morphologically. Its breeder only used it as a crossing partner. The variety New Golden Giant, bred using the pedigree method from the original population of Golden Giant, is again not identical to the American Golden Giant. The varieties Mindszentpusztai Yellow Dent and Szegedi Yellow Dent were developed using the same method from Chester Leaming and Funk Yellow Dent, respectively. The variety Illinois Champion had been maintained by farmers in Hungary over a long period. It probably arose from the Reid Yellow Dent variety exhibited in Illinois in 1891 by James Reid, which won a Grand Prize at the World Fair in Chicago two years later. The most popular dent variety of the period was

Garabosi Golden Dent, the origin of which is largely unknown. It enjoyed its greatest popularity among farmers of German extraction in Torontál County. The variety Garabosi Golden Dent is probably related to the Many-Rowed Dents described by Leng et al. (1962), from which the variety Sidski Zlatni Zuban also originates (Southern Dent × Corn Belt Dent?).

The maize varieties of Eastern Central Europe include a surprisingly large number of varieties derived from planned crosses and having distinct variety character, but no name (Table 5). Exhibitors simply gave the names of the components used in the crosses (e.g. Garabosi Golden Dent × Old Hungarian Yellow Flint). Several of these varieties can be seen to have had the same pedigree. The occurrence of varieties with the same pedigree from various locations may indicate that these were commercially available.

Table 4
Dent varieties (Budapest, 1914)

No.	Name of variety	Frequency	
		No.	%
1	American Yellow Dent	103	23.5
2	Garabosi Golden Dent	55	12.6
3	Illinois Champion	49	11.2
4	Bánkúti Late Dent	37	8.4
5	White Dent	29	6.6
6	Mastodon	27	6.2
7	Golden Glows	27	6.2
8	Queen of the Prairie	20	4.6
9	Szalontai Dent	20	4.6
10	Iowa Goldmine	14	3.2
11	Poor Man's Corn	13	3.0
12	Fehérvári fillér	9	2.1
13	Livingstone Early Golden	6	1.4
14	Golden Tooth	5	1.1
15	Mercer	5	1.1
16	Southern Prolific	3	0.7
17	Red Dent	3	0.7
18	Kozma Dent	2	0.5
19	Golden Beauty	2	0.5
20	Leaming	2	0.5
21	Golden Howern	2	0.5
22	Silvermine	2	0.5
23	Funk Yellow Dent	1	0.2
24	New Golden Giant	1	0.2
	Total	*437*	*100.0*
	According to genetic background		
	Corn Belt Dent	342	78.3
	Southern Dent	95	21.7
	American	270	61.9
	Hungarian	167	38.1
	Total	*437*	*100.0*

Table 5

Open-pollinated maize varieties originating from planned crosses (Budapest, 1914)

No.	Pedigree	Frequency	
		No.	%
1	American Yellow Dent × Old Hungarian Yellow Flint	23	25.6
2	Mastodon × Old Hungarian Yellow Flint	8	8.9
3	Legkorábbi Székely × Alcsúti pignolettó	7	7.8
4	Mastodon × Illinois Champion	6	6.7
5	American Yellow Dent × Legkorábbi Székely	6	6.7
6	Queen of the Prairie × Putyi	5	5.6
7	Old Hungarian Yellow Flint × Putyi	5	5.6
8	Putyi × Legkorábbi Székely	4	4.4
9	Old Hungarian Yellow Flint × Cinquantino	4	4.4
10	Old Hungarian Yellow Flint × Alcsúti pignolettó	4	4.4
11	Garabosi Golden Dent × Lincoln	4	4.4
12	Lapusnyaki × Legkorábbi Székely	3	3.3
13	Illinois Champion × Bánsági Korai	3	3.3
14	Old Hungarian Yellow Flint × King Philip	2	2.2
15	American Yellow Dent × Poor Man's Corn	2	2.2
16	Bánkúti Late Dent × Muraközi	1	1.1
17	Queen of the Prairie × Legkorábbi Székely	1	1.1
18	Yellow Dutton × Cinquantino	1	1.1
19	Pennsylvania 8 rows × Vágvölgyi	1,	1.1
	Total	*90*	*100.0*

Among the flint varieties (Table 6) variants of Old Hungarian Yellow Flint, of Caribbean origin, were used most frequently in new, planned crosses. Hard-grained flints were often used, too, while King Philip and Pennsylvania 8-row, which belonged to the Northern Flints, were only found once each among the crossing partners used in the exhibited varieties.

Table 6

Flint components used to develop maize varieties of hybrid origin (Budapest, 1914)

No.	Flint components used to develop varieties	Frequency	
		No.	%
1	Old Hungarian Yellow Flint	52	49.1
2	Legkorábbi Székely	21	19.8
3	Putyi	14	13.2
4	Alcsúti pignolettó	11	10.4
5	Cinquantino	5	4.7
6	Lapusnyaki	1	0.9
7	King Philip	1	0.9
8	Pennsylvania 8 rows	1	0.9
	Total	*106*	*100.0*

The dent varieties used to develop new varieties can be divided into Corn Belt Dents and Southern Dents (Table 7). In many cases the names of the dent varieties used in the 71 pedigrees differ from the originals (the users probably only knew them from hearsay). The expressions American, American Dent and American Yellow Dent were probably used to designate the same varieties as those known by their American names, and were used equally frequently. The most popular varieties used for the development of new varieties, presumably due to their production value, were Mastodon, Illinois Champion, Queen of the Prairie and Garabosi Golden Dent.

Table 7

Dent components used to develop maize varieties of hybrid origin (Budapest, 1914)

No.	Dent components used to develop varieties	Frequency	
		No.	%
1	American Yellow Dent	31	43.6
2	Mastodon	14	19.7
3	Illinois Champion	9	12.7
4	Queen of the Prairie	6	8.5
5	Garabosi Golden Dent	4	5.6
6	Lincoln	4	5.6
7	Poor Man's Corn	1	1.4
8	Bánkúti Dent	1	1.4
9	Yellow Dutton	1	1.4
	Total	*71*	*100.0*

Among the varieties derived from crosses, dent × flint combinations were the most popular (Table 8). The flint × flint pedigree was also unusually popular in Eastern Central Europe, primarily for the development of varieties with better yield potential and hard or semi-hard kernels suitable for human consumption. Such hybrids were possible because the Eastern Central European flints exhibited great genetic and morphological variability; there were at least 4 races of flints, which combined well to give excellent hybrids (these included the Óvári 1 variety hybrid registered in 1953: Mindszentpusztai White × Martonvásári F.B.). Corn Belt Dents and Southern Dents were also crossed (Illinois Champion × Mastodon) to develop high-yielding dent varieties (e.g. Garabosi Golden Dent).

Discussion

Earlier results (Hadi, 2004; 2005a; b) suggest that the open-pollinated maize varieties developed in Eastern Central Europe and the maize hybrids that became popular later had a genetic background different from that of genotypes from the North American Corn Belt and from other regions of Europe. In the continental climate of this region the vegetation period is shorter and the summer hotter and drier. By contrast the spring and autumn are cooler and wetter. The weather warms up more slowly in spring, and there are often early autumn frosts, while the cool, wet weather favours the spread of ear and stalk fusarium. Cold tolerance at germination, a short vegetation period and rapid maturing with low grain moisture at harvest are thus of special importance in Eastern Central Europe. The fact that 20–30% of the maize yield was used for human consumption (as porridge) also had a great influence on the variety structure.

For the above reasons, the production of new open-pollinated maize varieties began as long ago as the 1850s and 1860s, reaching a peak in 1900–1930. It was this peak of breeding activity that finalised the genetic variability of maize in Eastern Central Europe. Few American open-pollinated varieties were introduced into the gene pool between the two world wars.

As the result of the variety registration system, information is available for the vast majority of the maize varieties sold commercially regarding their origin, breeder, and economic value compared with rival varieties under contemporary agronomic conditions. Breeding by farmers was generally based on the on-farm maintenance of improved varieties or of varieties introduced from abroad. Landraces are the product of such reproduction. As is clear from the material exhibited at the Maize Exhibition in 1914, farmers breeding new varieties often made crosses or took advantage of the variability arising due to accidental mixing, as the majority of distinct maize varieties at the time had been developed through a process of inbreeding (selection for homogeneity, pedigree selection). Before new varieties could be developed it was necessary to enrich the initial sources. The new varieties bred by farmers were usually only grown locally.

Table 8
Pedigree types used most frequently (Budapest, 1914)

No.	Pedigree type	Frequency	
		No.	%
1	Corn Belt Dent × Central European soft flint	28	31.1
2	Central European soft flint × Central European soft flint	20	22.2
3	Corn Belt Dent × Central European hard flint	12	13.3
4	Southern Dent × Central European hard flint	8	8.9
5	Central European hard flint × Central European hard flint	7	7.8
6	Corn Belt Dent × Corn Belt Dent	6	6.7
7	Corn Belt Dent × Southern Dent	6	6.7
8	Central European soft flint × Northern Flint	3	3.3
	Total	*90*	*100.0*
	dent × flint	48	53.3
	flint × flint	30	33.3
	dent × dent	12	13.3
	Total	*90*	*100.0*

In the course of breeding, farmers carried out crosses to produce variety hybrids. On occasion the crosses were repeated, by sowing the components in alternate rows and removing the tassels from the female rows. In most cases mass selection was carried out on the variety population, leading, after selection for homogeneity, to the development of new varieties. A contemporary writer gives a clear illustration of this process: "The farm under my management produced an excellent crop of Yellow Hungarian × Florentini maize in 1911, as regards both quality and quantity, encouraging me to improve this cross, that had proved its worth under local conditions, by selecting the seed" (Nagy, 1913). It is impossible to decide, on the basis of the information provided, which of the varieties exhibited in Budapest in 1914 were true variety hybrids and which were new varieties developed by selection. Both groups are morphologically constant. The identical pedigrees entered from different villages suggest that the majority of these were true, morphologically constant varieties developed by selection for homogeneity.

The principles, techniques and methods required for the development and sale of true variety hybrids were not elaborated in Hungary until the 1940s (Berzsenyi-Janosits, 1951).

The utilisation of variety hybrids through repeated reproduction from farmer's seed probably began in Eastern Central Europe with the development of the first maize varieties of hybrid origin (Szentkirályi, 1877; Lubinszky, 1901; Hadi, 2005a). A considerable proportion of the 90 varieties that had no distinct variety name, but had known crossing components and were morphologically stable, and the 476 samples of mixed variety, generally excluded because they lacked constant, distinct variety traits, made up almost 30% of the exhibits. The large-scale use of farmer's seed to sow a second or third generation of variety hybrids was not reported either in the Corn Belt or in other regions of Europe, so it appears that this was a maize production practice peculiar to Eastern Central Europe and particularly popular in the southern counties (Torontál, Bácsbodrog, Temes) that later became part of Yugoslavia. This large-scale reproduction and variety improvement of advanced generations of variety hybrids by farmers may also serve as an explanation for the appearance of an exceptionally large number of open-pollinated varieties of hybrid origin in Eastern Central Europe, as noted by Leng et al. (1962), Pavlicic (1971), Trifunovic (1978) and Radovic and Jelovac (1995).

References

Agr. Z. (1855): Néhány kukorica fajnak aránylagos termékenysége. (Relative fertility of maize species.) *Gazdasági Lapok,* **7**, 281–283.

Barna, B. (1904): A kukorica nemesítése. (The breeding of maize). *Erdélyi Gazda,* **36**, 206–207.

Baross, L. (1916): A tengeri nemesítéséről. (On the improvement of maize.) *Köztelek*, **26**, 2–4.

Berzsenyi-Janosits, L. (1951): A kukoricatermés növelése fajta heterózissal. (Improvements in maize yields through variety heterosis.) *Magyar Mezőgazdaság*, **6**, 7–8.

Brandolini, G. A. (1968): European races of corn. *Proc. Corn and Sorghum Ind. Res. Conf.*, **24**, 36–48.

Brandolini, G. A. (1971): Preliminary report on South-European and Mediterranean maize germplasm. pp. 108–116. In: Kovács, I. (ed.), *Proc. Of the Fifth Meeting of the Maize and Sorghum Section of Eucarpia*. Akadémiai Kiadó, Budapest

Butujás, G. (1912): *Hazánkban termő fontosabb tengeri félék magjainak alak és alaktana, gazdasági értékükre való tekintettel*. (Seed morphology of the major maize varieties grown in Hungary, with special regard to their economic value. Thesis.) Stief Jenő és társa Könyvkiadó, Kolozsvár, 52 p.

Cserháti, S. (1895): Jelentés a M. Kir. Növénytermesztési Kísérleti Állomás 1894. évi működéséről. (1894 Annual Report from the Hungarian Royal Crop Production Station.) Magyaróvár, Czéh Sándor féle Könyvnyomda, pp. 131–143.

Csiktapolcai-Lázár, L. (1899): Egy pár szó az én "lapusnyaki" tengerimről és a vele tett kísérletekről. (A few words on my "lapusnyaki" maize and on experiments on it.) *Gazdasági Lapok*, **51**, 108–109.

Dudás, A. (1926): A Gyérei féle nemesített fehér lófogú tengeri. (Gyérei improved white dent variety.) *Köztelek*, **35**, 78–79.

Fabricius, E. (1914): *I. Országos kukorica kiállítás szakkatalógusa*. (Catalogue of the lst National Maize Exhibition.) Országos Magyar Gazdasági Egyesület (Pátria) Kiadója. Budapest, 127 p.

Falb, L. (1923): A zalaszentgróti nemesített sokcsövű pignolettó tengeri. (Improved prolific pignolettó from Zalaszentgrót.) *Köztelek*, **33**, 209–210.

Fleischmann, R. (1913a): A rumai uradalom tengeri nemesítő eljárása. (Maize breeding method followed on the Ruma estate.) *Köztelek*, **23**, 1694–1697.

Fleischmann, R. (1913b): A tengeri cső alakulásának jelentősége a tengeri nemesítésénél. (Importance of ear formation in maize breeding.) *Köztelek*, **23**, 3012–3013.

Grábner, E. (1913): A sokcsövű tengeri fajta létesítése nemesítés útján. (Development of a prolific maize variety through breeding.) *Gazdasági Lapok*, **65**, 850–851.

Grábner, E. (1916): Az 1916. évben államilag törzskönyvelt és elismert tengeri fajták. (Maize varieties state registered in 1916.) *Köztelek*, **26**, 1887–1889.

Grábner, E. (1917): A bánkuti nemesített kukorica fajták 1917. évi terméseredményei. (1917 yield figures for maize varieties bred in Bánkút.) *Köztelek*, **27**, 2134–2135.

Grábner, E. (1922): A magyar tengeri fajták. (Hungarian varieties of maize.) *Köztelek*, **32**, 280–281.

Grábner, E. (1929): Az 1929. évben állami fajtaelismeréssel hitelesített magyar tengeri és burgonya fajták. (Hungarian-bred maize and potato varieties state registered in 1929.) *Köztelek*, **39**, 2003.

Grábner, E. (1908): *A gazdasági növények nemesítése*. (Breeding of Crop Plants.) Az Országos Magyar Gazdasági Egyesület (Pátria) Könyvkiadó Vállalata, Budapest, pp. 168–180.

Hadi, G. (2004): Maize varieties grown in Eastern Central Europe between 1938 and 1983. *Acta Agron. Hung.*, **52**, 421–438.

Hadi, G. (2005a): Effect of popcorn varieties from the Andes on the development of the early, hard-grained gene pool in Central Europe. *Acta Agron. Hung.*, **53**, 109–118.

Hadi, G. (2005b): Contribution of the breeding methods used by Rudolf Fleischmann to the development of the Ruma maize heterosis source. *Cer. Res. Comm.*, **33**, 509–516.

Hadi, G., Illés, O., Szőke, C. (2003a): A rumai gén-pool kialakulása és szerepe a közép-európai hibridkukorica nemesítésben. (Development and role of the Ruma gene pool in hybrid maize breeding in Central Europe.) pp. 133–139. In: Marton, L. C., Árendás, T. (eds.), *50 éves a magyar hibridkukorica*. Martonvásár.

Hadi, G., Marton L. C., Szundy, T., Kovács, I., Pintér, J., Dolinka, B. (2003c): A Mindszentpusztai sárga lófogú kukorica fajta és a belőle származó vonalak szerepe az európai és magyar hibridkukorica nemesítésben. (Role of the maize variety Mindszentpusztai Yellow Dent and the lines derived from it in European and Hungarian hybrid maize breeding.) pp. 141–145. In: Marton, L. C., Árendás, T. (eds.), *50 éves a magyar hibridkukorica*. Martonvásár.

Hadi, G., Marton, L. C., Szundy, T., Kovács, I., Pintér, J., Dolinka, B. (2004): Contribution made by the maize variety Mindszentpusztai Yellow Dent (MYD) to the birth of hybrid maize in Hungary and in Europe as a whole. *Cer. Res. Comm.*, **32**, 159–166.

Hadi, G., Szőke, C., Illés, O. (2003b): A közép-európai soksorú flint gén pool kialakulása. (Development of the Central European early, multi-rowed flint maize gene pool.) pp. 147–154. In: Marton, L.C., Árendás, T. (eds.), *50 éves a magyar hibridkukorica*. Martonvásár.

Jánossy, A., Komlóssy, G., Mórász, S., Taróczy, H. (1957): *Magyar kukoricafajták és termesztésük*. (Hungarian maize varieties and their production.) Mezőgazdasági Kiadó, Budapest, 143 p.

Kárász, P. (1921): Sokcsövű páduai tengeri. (Prolific maize from Padua.) *Köztelek*, **31**, 672.

Kenyeres, L. (1913): Új fajta kukorica. (A new variety of maize.) *Magyar Földműves*, **47**, 745–746.

Leng, E., Tavcar, R. A., Trifunovic, V. (1962): Maize of Southeastern Europe and its potential in breeding programs elsewhere. *Euphytica*, **11**, 263–272.

Lubinszky, E. (1901): A Putyi kukorica. (The maize variety Putyi.) *Köztelek*, **11**, 1884–1885.

Lubinszky, E. (1903): A Putyi kukorica 1902. évi termése. (The 1902 yield of the maize variety Putyi.) *Köztelek*, **13**, 4–6.

Marton, L. C., Németh, J. (2003): A kukorica. (Maize.) In: Koháry, E. (ed.) *Eleven örökség. Kenyér és kásanövények a Kárpát medencében.* (Living heritage. Bread and porridge crops in the Carpathian Basin.) Agroinform Kiadó, Budapest, pp. 67–79.

Nagy, B. (1913): A kukorica vetőmag kiválasztásának haszna. (The profitability of selecting maize seed.) *Köztelek*, **23**, 866–867.

Pap, E. (1929): A nemesített mindszentpusztai korai fehér tengeri. (Improved early white maize variety from Mindszentpuszta.) *Köztelek*, **39**, 384–385.

Pavlicic, J. (1971): Contribution to a preliminary classification of European open-pollinated maize varieties. Pp. 93–107. In: Kovács, I. (ed.), *Proc. of the Fifth Meeting of the Maize and Sorghum Section of Eucarpia*. Akadémiai Kiadó, Budapest.

Péterfy, T. (1912a): Magyar kukorica fajták. Lapusnyaki kukorica. (Hungarian maize varieties. The variety Lapusnyaki.) *Köztelek*, **22**, 985.

Péterfy, T. (1912b): Magyar kukorica fajták. A bánkúti kukorica. (Hungarian maize varieties. The variety Bánkúti.) *Köztelek*, **22**, 1124–1125.

Péterfy, T. (1912c): Magyar kukorica fajták. A Putyi kukorica. (Hungarian maize varieties. The variety Putyi.) *Köztelek*, **22**, 1195–1196.

Péterfy, T. (1912d): Magyar kukorica fajták. A Székely kukorica. (Hungarian maize varieties. The variety Székely.) *Köztelek*, **22**, 1273.

Péterfy, T. (1912e): Magyar kukorica fajták. Alcsúti kukorica. (Hungarian maize varieties. The variety Alcsúti maize.) *Köztelek*, **22**, 1672–1673.

Péterfy, T. (1912f): Magyar kukorica fajták. Fehérvári kukorica. (Hungarian maize varieties. The variety Fehérvári.) *Köztelek*, **22**, 1860.

Péterfy, T. (1913): A legkorábbi magyar kukorica. (The earliest Hungarian maize varieties.) *Magyar Földműves*, **4**, 503–504.

Radovic, G., Jelovac, D. (1995): Identification of heterotic pattern in Yugoslav maize germplasm. *Maydica*, **40**, 223–227.

Röszler, K. (1907): Tengeri termesztési kísérletek. (Production trials on maize.) *Kísérletügyi Közlemények*, **10**, 394–412.

Surányi, J. (1914): A kukorica származása és helye a világ mezőgazdaságában. (Origin of maize and its place in world agriculture.) *Gazdasági Lapok*, **66**, 330–332.

Szentkirályi, Á. (1877): Korán érő törökbúza. (Early maturing maize.) *Erdélyi Gazda*, **9**, 195.

Szentkirályi, Á. (1881): A koraérő székely tengeri. (Early maturing maize from the Székely region.) *Gyakorlati Mezőgazda*, **10**, 68.

Taróczy, H. (1952): Kukorica (*Zea mays*) nemesített növényfajtákkal végzett országos kísérletek eredményei 1951. (Results of national experiments on improved varieties of maize (*Zea mays*) 1951.), Országos Vetőmagvizsgáló Int., Budapest, pp. 91–117.

Taróczy, H. (1954): Kukorica. Nemesített növény fajtákkal végzett országos fajtakísérletek eredményei. (Maize. Results of national experiments on improved plant varieties.) Országos Vetőmagvizsgáló Int., Budapest, pp. 239–257.

Timár, J. (1913): Szemesen vagy csövesen vásároljunk kukorica vetőmagot? (Should we buy maize seed loose or on the ear?) *Köztelek*, **23**, 131–132.

Trifunovic, V. (1978): Maize production and maize breeding in Europe. pp. 41–58. In: Walden, B. D. (ed.), *Maize Breeding and Genetics*. John Wiley and Sons, New York, Chichester, Brisbane, Toronto.

Villax, Ö., Surányi, J. (1932): Varieties of corn in Hungary. Magyaróvár, 64 p.

Zathureczky, K. (1912): Temesvári tengeri kiállítás. (Maize exhibition in Temesvár.) *Gazdasági Lapok*, **62**, 828–830.

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PHOTOSYNTHETIC ATTRIBUTES AND GRAIN YIELD OF PEARL MILLET (*Pennisetum glaucum* (L.) R. Br.) AS INFLUENCED BY THE APPLICATION OF COMPOSTED COIR PITH UNDER RAINFED CONDITIONS

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A field experiment was conducted at the Eastern Block, Department of Central Farm, Tamil Nadu Agricultural University, Coimbatore, India, on medium black soils during the North Eastern Monsoon Season (October–January) of 2002. The experiment was laid out in a randomized block design (RBD) with varying combinations of organic and inorganic sources along with a control and the recommended dose of fertilizers. The results revealed that the number of tillers per plant was enhanced by the application of a combination of organic and inorganic sources. The leaf area index (LAI) increased up to 60 days after sowing and thereafter declined. Leaf area duration (LAD) and crop growth rate (CGR) were reduced at maturity. Treatments receiving 50% N from yeast sludge composted coir pith (YCCP) with $ZnSO_4$ and 50% inorganic N gave a significantly higher number of tillers, LAI and CGR compared to the control. The dry matter accumulation differed in all the stages, but higher values were recorded in this same treatment. Thus, due to the higher number of tillers, higher LAI, CGR and greater dry matter accumulation, treatment with 50% N from YCCP with $ZnSO_4$ and 50% inorganic N (T_{10}) produced the highest grain yield (2185 kg ha^{-1}), which was on par with 50% N from pleurotus composted coir pith (PCCP) with $ZnSO_4$ and 50% inorganic N (2103 kg ha^{-1}).

Key words: yeast sludge composted coir pith (YCCP), pleurotus composted coir pith (PCCP), zinc sulphate ($ZnSO_4$), photosynthetic attributes, grain yield, pearl millet

Introduction

In India, about 143 million ha of the total geographical area of 329 million ha is under cultivation. Rainfed agriculture is carried out on around 97 million ha of the cultivated area. This supports 40% of India's total population and contributes 44% to the national food basket. About 68% of the total cropped area in India is under rainfed cultivation. This area contributes 40% of cereals, 75% of total pulses, 75% of total oil seeds and 65% of total cotton production. In Tamil Nadu, 60% of the cultivated area is rainfed. Since the available water resources, both from surface and subsurface waters, are fully utilized, the scope for bringing additional areas under irrigation is negligible. Hence, research efforts are focused on enhancing the productivity of the rainfed area to meet the food requirements of the growing population.

India is the largest pearl millet (*Pennisetum glaucum* (L.) R. Br.) growing country, contributing 42% of total world production. In India, pearl millet is predominantly cultivated as a rainfed crop under diverse soil and climatic conditions and is an indispensable arid zone crop. In India, it is cultivated on an area of 9.55 million hectares with an annual production of 8.35 million tonnes and a productivity of 875 kg ha^{-1} (Anonymous, 2003). The pearl millet area in Tamil Nadu is 125,093 hectares with an annual production of 152,970 tonnes and a productivity of 1223 kg ha^{-1} (Anonymous, 2002).

The major constraints in dryland agriculture are the inadequacy of soil moisture and poor soil fertility status. The experimental results indicated that the application of organic manure improves not only soil fertility status but also the water-holding capacity of the soil, especially under dryland cultivation.

The land, which is at the mercy of rainfall, is not only thirsty, but also hungry. The estimated nutrient removal by all dryland crops amounts to 7.4 million tonnes (excluding secondary and micronutrients). Drylands receive approximately 10% of the total nutrient use in the country, constituting about 1.4 million tonnes. There remains a net negative balance of about 6.0 million tonnes (Venkata Lakshmi, 2001). The gap will widen further as time proceeds, if adequate steps are not taken to replenish the soil fertility by means of additional nutrition. The alarming trend in environmental pollution emphasizes the need to use organic manures to sustain soil productivity. The prevention of environmental pollution and the increasing cost of chemical fertilizers warrant an integrated nutrient management approach in agriculture.

Limitations in the availability of conventional manures like farmyard manure (FYM) can be overcome through the exploitation of new sources of organic matter like coir pith. Coir pith, a by-product of the coir industry, constitutes about 70% of the coconut husk and 7.5 million tonnes of coir pith are produced per annum in India (Kamaraj, 1994). In Tamil Nadu, nearly 0.2 million tonnes of coir pith are available annually (Parasuraman et al., 2002). The spongy structure of coir pith facilitates the retention of water in soil and the slow release of nutrients. Under dryland conditions the combined use of organic manures and inorganic fertilizers is essential for sustaining crop yields owing to the need for greater moisture retention, besides the addition of nutrients and improvements in soil conditions.

The use of composted coir pith in agriculture is gaining popularity and the use of *Pleurotus sajor-caju* for composting is general practice. Yeast sludge is a solid waste produced by alcohol distilleries at a rate of 2 t day^{-1} (Rajannan et al., 2002). It is also produced as waste by the sugar industry. Information on the effect of yeast sludge composted coir pith (YCCP) on the productivity of pearl millet under dryland conditions is scanty. There is also little or no information available regarding the effect of composted coir pith on the photosynthetic attributes of pearl millet. Hence, the present study was planned and carried out to assess the effect of organic sources, namely composted coir pith, and their combination with inorganic sources on the photosynthetic attributes and grain yield of a pearl millet hybrid.

Materials and methods

The field experiment was conducted during the North East Monsoon season (October–January) of 2002–2003 at the Eastern Block, Department of Central Farm, Tamil Nadu Agricultural University, Coimbatore. The experimental site is situated at 11° N latitude, 77° E longitude and at an altitude of 426.7 m above mean sea level. The soil was sandy clay loam in texture with a pH of 8.1. The soil was low in organic carbon (0.31%), low in available nitrogen (215 kg N ha^{-1}), medium in available phosphorus (17.2 kg P_2O_5 ha^{-1}) and high in available potassium (470 kg K_2O ha^{-1}), as estimated using the chromic acid wet digestion method (Walkley and Black, 1934), the alkaline permanganate method (Subbiah and Asija, 1956), Olsen's method (Olsen et al., 1954) and the flame photometer method (Stanford and English, 1949), respectively. Pearl millet hybrid CoHCu8, with a field duration of 80 days, was used in the trial.

The experiment was laid out in a randomized block design (RBD) with three replications on a gross plot size of 5 m × 4 m and a net plot size of 4.65 × 2.25 m. The experiment involved ten treatments (see Tables) with various combinations of organic and inorganic sources. The organic source used was coir pith, a by-product of the coir industry. Coir pith compost was prepared by two methods. In the Pleurotus method of composting, an area 5 m long and 3 m wide was marked off near the experimental plot. One hundred kg of raw coir pith was spread uniformly over the marked area and a layer of spawn, *Pleurotus sajor-caju* was spread over it uniformly. Above this layer another 100 kg of coir pith was spread uniformly and over this one kg of urea was applied. The process was repeated by sandwiching the *Pleurotus sajor-caju* and urea alternatively to a height of 1 m. Sprinkling with water was done twice a week for 30 days. During the composting process the moisture was maintained at 50%. Coir compost was also prepared using yeast sludge. The coir waste was sieved so as to remove all the fibrous materials. To each tonne of coir waste, 200 kg of yeast sludge and 10 kg of rock phosphate was added and mixed thoroughly. After mixing, the materials were formed into a heap. The moisture content of the compost was maintained at 60%. Within a period of 4–5 days the temperature in the heap rose to 50–60°C. This was measured using a thermometer or by touching with the fingers. When the temperature dropped to below 50°C, the heap was stirred and moistened with water to maintain 60%. The colour, smell, temperature, texture and heaviness of the compost was observed carefully. Watering was done four times a week. Coir pith and yeast sludge formed a matured compost within a period of 50 days. Zinc sulphate @ 25 kg ha^{-1} was added with the composted coir pith at the time of application.

Based on N content, the quantity of composted coir pith was fixed to supply the quantity of N required by the treatment schedule. Pleurotus composted coir pith (PCCP) contains 0.81% N and was applied to the field at rates of 5.6 t ha^{-1} and 2.8 t ha^{-1} to supply 100 and 50% N, respectively. Yeast sludge composted coir pith contains 0.92% N and was applied to the field at rates of 4.9 t ha^{-1} and 2.45 t ha^{-1} to supply 100 and 50% N. Composted coir pith was spread over the respective treatment plots and incorporated immediately into the soil with a spade. It was applied on an N equivalent basis to contribute the required N, as per the treatment schedule. The inorganic fertilizers urea and single superphosphate were applied to supply the required quantities of N and P, respectively. All the inorganic fertilizers were applied basally. Line sowing was carried out by adopting a spacing of 45 cm between rows and 15 cm between plants. Gap filling and thinning were done 13 and 20 days after sowing (DAS), respectively. Hoeing and weeding were carried out 20 and 45 DAS. Plant protection measures were carried out to control pests. The crop was harvested when it attained maturity.

To record various biometric observations on pearl millet, a sample consisting of five plants was selected at random from each net plot and tagged for observations on plant height, tillers, leaf area index (LAI) and dry matter production at 30 and 60 DAS and at harvest. Plant height was measured from ground level. The number of tillers was counted. For LAI, plants were cut close to the ground and leaves from the plants were fed into a LI-COR LI-3100 Leaf Area Meter to record the leaf area. LAI was calculated using the formula of Williams (1946). Leaf area duration (LAD) and crop growth rate (CGR) were calculated using the formulae of Power et al. (1967) and Watson (1956), respectively. The samples were air dried and then oven dried at 60°C to constant weight. The weight of the oven dried samples was recorded and dry matter production was calculated and expressed in kg ha^{-1}. The data were subjected to statistical analysis (Panse and Sukhatma, 1978).

Results and discussion

Grain yield

Nitrogen management practices had a significant influence on the grain and stover yield of rainfed pearl millet (Table 1). The application of 50% N from YCCP with $ZnSO_4$ and 50% inorganic N (T_{10}) gave higher grain and stover yields than the other treatments and was on par with 50% N from PCCP with $ZnSO_4$ and 50% inorganic N (T_9). Treatment T_{10} resulted in 48 and 46% increases in the grain and stover yield, respectively, over the control. Kadalli et al. (2000) reported that the grain yield increase in maize was due to the application of coir pith @ 10 t ha^{-1} with 50% recommended NPK fertilizer. The application of YCCP and PCCP enhanced the soil nutrient status and increased nutrient uptake, thus increasing the crop yield. An increase in the grain and stover yields of pearl millet due to supplying 50% N from organic sources in conjunction with 50% recommended NPK was also reported by Tolanur and Badanur (2003). The increase in grain yield in T_{10} compared with 50% N from YCCP and 50% inorganic N (T_8), the recommended rate of NPK (T_2) and 50% N from PCCP and 50% inorganic N (T_7) was 12.2, 13.2 and 15.1%, respectively.

Photosynthetic attributes

Higher grain yield was mainly due to improvements in photosynthetic attributes in the above treatments. The photosynthetically active parameters of pearl millet, such as leaf area and LAI, number of tillers, DMP, etc., differed significantly in various treatments.

Table 1

Effect of composted coir pith on the grain and stover yield (kg ha^{-1}) of pearl millet

Treatments	Grain yield	Stover yield
T_1 – Control	1145	1984
T_2 – Recommended dose of fertilizers	1896	3146
T_3 – 100% N from PCCP	1403	2235
T_4 – 100% N from YCCP	1462	2386
T_5 – 100% N from PCCP with $ZnSO_4$	1588	2694
T_6 – 100% N from YCCP with $ZnSO_4$	1664	2830
T_7 – 50% N from PCCP + 50% inorganic N	1856	3038
T_8 – 50% N from YCCP + 50% inorganic N	1918	3204
T_9 – 50% N from PCCP with $ZnSO_4$ + 50% inorganic N	2103	3540
T_{10} – 50% N from YCCP with $ZnSO_4$ + 50% inorganic N	2185	3692
SEd	53.77	89.77
CD (P=0.05)	112.98	188.61

YCCP = yeast sludge composted coir pith; PCCP = *Pleurotus* composted coir pith

Number of tillers

The nitrogen management practices significantly influenced the number of tillers compared to the control (Table 2). The treatment involving 50% N from YCCP with $ZnSO_4$ and 50% inorganic N (T_{10}) gave a higher number of tillers (4.8) per plant, which was on par with 50% N from PCCP with $ZnSO_4$ and 50% inorganic N (T_9) and 50% N from YCCP and 50% inorganic N (T_8). The application of the recommended dose of inorganic fertilizer (T_2) led to a tiller number which was on par with 50% N from composted coir pith and 50% inorganic N.

Composted coir pith (CCP) with $ZnSO_4$ and inorganic N application increased the tiller number. The increase in available nitrogen induced better plant growth and improved physiological functions. The increase in the availability of phosphorus and its favourable effect on cell division, albumin formation and root development favoured tiller production (Munda et al., 1983). Treatments with 50% N from CCP and 50% inorganic N were comparable to the application of the recommended dose of inorganic fertilizer. These treatments gave a significantly higher number of tillers than CCP application alone. This emphasises the advantage of the partial substitution of inorganic N with organics to enhance the tiller number of pearl millet crops.

Leaf area index (LAI)

Among the different nitrogen management practices, the application of 50% N from YCCP with $ZnSO_4$ and 50% inorganic N (T_{10}) led to higher LAI at all stages (1.32, 3.10 and 1.15 at 30, 60 DAS and at harvest, respectively) and was on par with 50% N from PCCP with $ZnSO_4$ and 50% inorganic N (T_9). The LAI recorded for the recommended inorganic fertilizer application (T_2) was on par with 50% N from composted coir pith and 50% inorganic N application (T_8 and T_7).

Table 2

Effect of composted coir pith on total tiller number of pearl millet

Treatments	Total No. of tillers at harvest
T_1 – Control	3.2
T_2 – Recommended dose of fertilizers	4.5
T_3 – 100% N from PCCP	3.8
T_4 – 100% N from YCCP	3.9
T_5 – 100% N from PCCP with $ZnSO_4$	4.1
T_6 – 100% N from YCCP with $ZnSO_4$	4.2
T_7 – 50% N from PCCP + 50% inorganic N	4.5
T_8 – 50% N from YCCP + 50% inorganic N	4.5
T_9 – 50% N from PCCP with $ZnSO_4$ + 50% inorganic N	4.7
T_{10} – 50% N from YCCP with $ZnSO_4$ + 50% inorganic N	4.8
SEd	0.13
CD (P=0.05)	0.28

YCCP = yeast sludge composted coir pith; PCCP = *Pleurotus* composted coir pith

LAI is a reflection of the physiological efficiency of the crop for better photosynthesis and is also an indicator of the sufficiency or deficiency of nitrogenous nutrition. One of the principle factors influencing canopy net photosynthesis is LAI (Hansen, 1972), which increased rapidly and linearly up to the end of blooming, attaining a maximum at 60 DAS. Thereafter, LAI declined due to the abscission of the lower leaves. Nitrogen levels influenced the LAI significantly (Table 3).

A significant increase in LAI was observed in treatments involving 50% N from YCCP (T_{10}) or PCCP (T_9) with $ZnSO_4$ and 50% inorganic N. The rapid mineralisation and increased availability of plant nutrients in these treatments enhanced the LAI (Saxena et al., 2001). $ZnSO_4$ application with CCP significantly influenced the LAI. Zinc is a component of enzymes essential for the assimilation of N and helps in the formation of chlorophyll (Singh and Vyas, 1998).

Table 3

Effect of composted coir pith on the leaf area index of pearl millet

Treatments	30 DAS	60 DAS	Harvest
T_1 – Control	0.82	2.56	0.67
T_2 – Recommended dose of fertilizers	1.24	2.69	0.86
T_3 – 100% N from PCCP	1.18	2.66	0.92
T_4 – 100% N from YCCP	1.16	2.64	0.94
T_5 – 100% N from PCCP with $ZnSO_4$	1.18	2.68	0.98
T_6 – 100% N from YCCP with $ZnSO_4$	1.17	2.66	0.98
T_7 – 50% N from PCCP + 50% inorganic N	1.21	2.80	1.04
T_8 – 50% N from YCCP + 50% inorganic N	1.23	2.82	1.06
T_9 – 50% N from PCCP with $ZnSO_4$ + 50% inorganic N	1.31	3.00	1.14
T_{10} – 50% N from YCCP with $ZnSO_4$ + 50% inorganic N	1.32	3.10	1.15
SEd	0.04	0.08	0.03
CD (P=0.05)	0.08	0.18	0.07

YCCP = yeast sludge composted coir pith; PCCP = *Pleurotus* composted coir pith

Leaf area duration (LAD)

LAD is a measure of the duration of the photosynthetic apparatus and is an important factor for the growth and development of the crop (Evans, 1975). Composted coir pith treatments with $ZnSO_4$ and inorganic N application significantly increased LAD over other treatments (Table 4). This might be due to the enhancement of LAI by these treatments. Similar findings were reported by Devarani (1996).

Crop growth rate (CGR)

CGR is a linear function of intercepted irradiance and the maintenance of higher LAI, which has a positive effect on dry matter production (Shibles and Webber, 1966). The crop growth rate was significantly influenced by the various nitrogen management practices (Table 5). The application of 50% N from YCCP with $ZnSO_4$ and 50% inorganic N (T_{10}) gave significantly higher CGR (19.87 g m^{-2} day^{-1}) than the other treatments but was on par with 50% N from PCCP with $ZnSO_4$ and 50% inorganic N (T_9) in the first stage. In treatments involving 50% N from YCCP or PCCP with 50% inorganic N (T_8 and T_7) CGR was comparable with that of the recommended dose of fertilizer application (T_2) in both stages. The application of composted coir pith to supply 100% N with $ZnSO_4$ (T_6 and T_5) significantly enhanced the CGR compared to CCP application without $ZnSO_4$ (T_3 and T_4) application. CCP with $ZnSO_4$ and inorganic N application improved the CGR by enhancing the LAI and LAD of the crop. A significant effect on physiological parameters such as CGR from the combined application of organic and inorganic nutrients was also reported by Saxena et al. (2001).

Table 4

Effect of composted coir pith on the leaf area duration (days) of pearl millet

Treatments	30 – 60 DAS	60 DAS – harvest
T_1 – Control	50.7	48.5
T_2 – Recommended dose of fertilizers	59.0	53.3
T_3 – 100% N from PCCP	57.6	53.7
T_4 – 100% N from YCCP	57.0	53.7
T_5 – 100% N from PCCP with $ZnSO_4$	57.9	54.9
T_6 – 100% N from YCCP with $ZnSO_4$	57.5	54.6
T_7 – 50% N from PCCP + 50% inorganic N	60.2	57.6
T_8 – 50% N from YCCP + 50% inorganic N	60.8	58.2
T_9 – 50% N from PCCP with $ZnSO_4$ + 50% inorganic N	64.7	62.1
T_{10} – 50% N from YCCP with $ZnSO_4$ + 50% inorganic N	66.3	63.8
SEd	1.83	1.73
CD (P=0.05)	3.84	3.64

YCCP = yeast sludge composted coir pith; PCCP = *Pleurotus* composted coir pith

Table 5

Effect of composted coir pith on the crop growth rate (g m^{-2} day^{-1}) of pearl millet

Treatments	30 – 60 DAS	60 DAS – harvest
T_1 – Control	11.71	7.83
T_2 – Recommended dose of fertilizers	17.50	11.26
T_3 – 100% N from PCCP	13.29	9.68
T_4 – 100% N from YCCP	14.02	9.37
T_5 – 100% N from PCCP with $ZnSO_4$	15.28	10.10
T_6 – 100% N from YCCP with $ZnSO_4$	15.89	10.20
T_7 – 50% N from PCCP + 50% inorganic N	17.12	10.96
T_8 – 50% N from YCCP + 50% inorganic N	17.88	11.02
T_9 – 50% N from PCCP with $ZnSO_4$ + 50% inorganic N	19.32	11.58
T_{10} – 50% N from YCCP with $ZnSO_4$ + 50% inorganic N	19.87	11.46
SEd	0.50	0.32
CD (P=0.05)	1.06	0.68

YCCP = yeast sludge composted coir pith; PCCP = *Pleurotus* composted coir pith

Dry matter production (DMP)

Treatments involving 50% N from YCCP or PCCP with $ZnSO_4$ and 50% inorganic N (T_{10} and T_9) led to higher DMP than other treatments at all stages (Table 6). This might be due to the favourable influence of these treatments on growth characters such as plant height, LAI, LAD and CGR, which was reflected in DMP. The increase in DMP due to CCP with 50% N application in soybean was reported by Logama Devi (1997).

$ZnSO_4$ with CCP application increased the DMP at all stages due to the increase in the nutrients available to the crops. The significant interaction between N and Zn enhanced the N availability in linseed crops, as reported by Mishra and Singh (1996). Singh and Vyas (1998) proved that the increase in dry matter production was due to the application of Zn at various levels.

The present investigation thus revealed that pearl millet responds very well to composted coir pith application along with inorganic fertilizer N and the micronutrient zinc under rainfed conditions. Treatment T_{10} produced an increase in the number of tillers, LAI and dry matter production compared with the other treatments and the control. The present study clearly indicated the effect of integrated nitrogen management on these photosynthetic attributes and its positive relationship with grain yield. The higher values of these characters led to the greater accumulation of photosynthates, which ultimately increased the grain yield of pearl millet.

Drastic increases in the price of chemical fertilizers and the environmental pollution caused by the increasing use of inorganic fertilizers have created interest in the use of organic wastes such as crop residues and agrobased industrial waste products. Coir pith is a by-product of the coir industry and is cheaply available in enormous quantities. Being an organic waste, its application improves the physical, chemical and biological properties of the soil.

Table 6

Effect of composted coir pith on the dry matter production (kg ha^{-1}) of pearl millet

Treatments	30 DAS	60 DAS	Harvest
T_1 – Control	354	3673	5892
T_2 – Recommended dose of fertilizers	572	5532	8724
T_3 – 100% N from PCCP	402	4169	6912
T_4 – 100% N from YCCP	428	4403	7058
T_5 – 100% N from PCCP with $ZnSO_4$	486	4818	7680
T_6 – 100% N from YCCP with $ZnSO_4$	508	5012	7904
T_7 – 50% N from PCCP + 50% inorganic N	564	5418	8525
T_8 – 50% N from YCCP + 50% inorganic N	586	5654	8778
T_9 – 50% N from PCCP with $ZnSO_4$ + 50% inorganic N	663	6140	9424
T_{10} – 50% N from YCCP with $ZnSO_4$ + 50% inorganic N	694	6326	9574
SEd	16.46	159.22	249.98
CD (P=0.05)	34.59	334.52	525.20

YCCP = yeast sludge composted coir pith; PCCP = *Pleurotus* composted coir pith

The addition of coir pith helps to conserve soil moisture. Hence, the partial substitution of these materials after composting will solve the above-mentioned problem. The moisture absorbing capacity of this material can be efficiently utilised under rainfed conditions to enhance the availability of soil moisture to the crops. Soil fertility can be sustained, crop productivity can be enhanced and the environment can be protected from pollution through the partial replacement of chemical fertilizers by organic manures like composted coir pith.

References

Anonymous (2002): *Season and Crop Report*. Directorate of Economics and Statistics, Government of Tamil Nadu.

Anonymous (2003): *Annual Report 2002–2003*. All-India Coordinated Pearl Millet Improvement Project. XXXVII, ICAR, New Delhi, p 7.

Devarani, N. (1996): *Nutrient and drought management packages for rainfed sorghum under vertisols*. M.Sc. Thesis, Tamil Nadu Agricultural University, Coimbatore, India.

Evans, L. T. (1975): The physiological aspects of crop yield. In: Evans., L. T. (ed.), *Crop Physiology – Some Case Histories*. Cambridge Univ. Press, London.

Hansen, W. R. (1972): *Net photosynthesis and evapotranspiration of field grown soybean canopies*. Ph.D. Thesis. Iowa State University Library, Ames.

Kadalli, G. G., Suseela Devi, L., Siddaramappa, R., Patil, C. R. (2000): Quality and efficiency of value added coirdust based compost. *J. Indian Soc. Soil Sci.*, **48**, 141–144.

Kamaraj, C. M. (1994): Exportable coir products in Tamil Nadu. *The Coconut Wealth,* **1**(6), 6–8.

Logama Devi, A. (1997): Influence of coirwaste on soil health and crop productivity. In: *Asian Soil Scenario and Fertility Management,* IFOAM, Bangalore, India. pp. 4–5.

Mishra, J., Singh, R. S. (1996): Effect of nitrogen and zinc on the growth and uptake of N and Zn by linseed. *J. Indian Soc. Soil Sci.,* **44,** 338–340.

Munda, G. C., Mahendra, P., Pandey, S. L. (1983): Effect of nitrogen and phosphorus on the yield, quality and yield attributing characters of pearl-millet. *Indian J. Agron.,* **28**, 332–338.

Olsen, S. R., Cole, C. V., Watanabe, F. S., Dean, L. A. (1954): Estimation of available phosphorus in soil by extraction with sodium carbonate. *U.S.D.A. Circ.* 939.

Panse, V. G., Sukhatme, P. V. (1978): *Statistical Methods for Agricultural Workers*. ICAR Publication, New Delhi.

Parasuraman, P., Mani, A. K., Suresh, M. (2002): Coirpith compost for rice-ragi cropping system of north western zone of Tamil Nadu. *Madras Agric. J.*, **89,** 369–370.

Power, J. E., Wills, W. O., Granes, D. L., Reickman, G. A. (1967): Effect of soil temperature, phosphorus and plant age on growth analysis of barley. *Agron. J.,* **59,** 231–234.

Rajannan, G., Udayasoorian, C., Maheswari, M., Thangavel, P., Selvaseelan, D. A., Thiyagarajan, T. M. (2002): Preparation of coir waste compost using yeast sludge. In: *Rapid Composting Technologies for Agricultural Wastes*. Department of Environmental Sciences, Tamil Nadu Agricultural University, Coimbatore, India.

Saxena, S. C., Manral, H. S., Chandel, A. S. (2001): Effect of inorganic and organic sources of nutrients on soybean (*Glycine max*). *Indian J. Agron.*, **46,** 135–140.

Shibles, R. M., Webber, C. R. (1966): Interruption of solar radiation and drymatter production by various soybean planting patterns. *Crop Sci.,* **6,** 55–59.

Singh, A., Vyas, A. K. (1998): Effect of nitrogen levels and zinc application on dry matter production and N, P and Zn content in maize. *Haryana J. Agron.,* **14,** 120–122.

Stanford, S., English, L. (1949): Use of flame photometer in rapid soil tests for potassium and sodium. *Agron. J.,* **4,** 446–447.

Subbiah, B. V., Asija. G. L. (1956): A rapid procedure for the estimation of available nitrogen in soil. *Curr. Sci.*, **25,** 259–260.

Tolanur, S. I., Badanur, V. P. (2003): Changes in organic carbon, available N, P and K under integrated use of organic manure, green manure and fertilizer on sustaining productivity of pearl millet – pigeonpea system and fertility of an inceptisol. *J. Indian Soc. Soil Sci.*, **51,** 37–41.

Venkata Lakshmi, K. (2001): *Integrated nutrient management for dryland fodder sorghum* (Sorghum bicolor *L. Moench*) – *chickpea* (Cicer arietinum *L.*) + *coriander* (Coriandrum sativum *L.*) *cropping system*. M.Sc. Thesis. Tamil Nadu Agricultural University, Coimbatore, India.

Walkley, A., Black, C. A. (1934): An estimation of different methods for determining soil organic matter and a proposed modification of the chromic acid titration method. *Soil Sci.*, **37,** 29–30.

Watson, D. J. (1956): Comparative physiological studies on the growth of field crops. I. Variations in net assimilation rate and leaf area between species and varieties and within and between years. *Ann. Bot.*, **2,** 41–76.

Williams, S. R. F. (1946): Methods of growth analysis. pp. 348–391. In: Sestack, Z., Catasky, J., Jouris, P. J. (eds.), *Plant Photosynthetic Production Manual Methods*. Drow. Jenk. N.U. Publishers, The Hague.

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EFFECT OF DIFFERENT COMBINATIONS OF INORGANIC NUTRIENTS AND FARMYARD MANURE ON THE SUSTAINABILITY OF A RICE–WHEAT–MUNGBEAN CROPPING SYSTEM

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A field study conducted for two years (1995–96 and 1996–97) at the Indian Agricultural Research Institute, New Delhi on a sandy clay loam soil showed that the application of NP increased the total grain production of a rice–wheat–mungbean cropping system by 0.5–0.6 t ha^{-1}, NK by 0.3–0.5 t ha^{-1} and NPK by 0.8–0.9 t ha^{-1} compared to N alone, indicating that the balanced use of primary nutrients was more advantageous than their imbalanced application. The application of farmyard manure (FYM) along with NPK further increased the total productivity of the rice–wheat–mungbean cropping system by 0.3–0.6 t ha^{-1}, the organic C by 0.13%, the available N by 10.7 kg ha^{-1}, the available P by 4.7 kg ha^{-1} and the available K by 15 kg ha^{-1} compared to NPK after two crop cycles of the system. The results of the present study thus indicate that integrated nutrient management involving FYM and NPK fertilizers is a must for the sustainability of a cropping system.

Key words: nitrogen, phosphorus, potassium, zinc, farmyard manure, productivity, nutrient uptake, soil fertility

Introduction

Rice-wheat cropping systems occupy about 22 million hectares in South-East Asia covering India, Pakistan, Nepal, Bangladesh and China (Fujisaka et al., 1994). In India this system is practised on 10.3 million hectares in the north-western part of the country and is considered as the backbone of the country's food security. However, in recent years concern has been expressed about the sustainability of the rice–wheat cropping system and the declining factor productivity of the fertilizer (lower response to applied fertilizer) (Yadav, 1998). As a result of this, farmers are applying more and more fertilizer to get the same level of yield as in the previous year, which is creating problems of groundwater contamination with nitrates (Singh et al., 1995). Hence, the supplementary and complementary role of organic manures in sustained production is becoming clear. Sharma et al. (1995) and Sharma and Prasad (1999) recommended sowing a short duration mungbean and incorporating its residues during the summer months, whereas Prasad and Mishra (2001) suggested FYM application at 10 t ha^{-1} in addition to recommended NPK doses to ensure the sustainability of the rice-wheat cropping system. The present study was therefore undertaken to study the effect of different combinations of inorganic nutrients and FYM on the productivity, nutrient uptake and soil fertility of a rice–wheat–mungbean cropping system.

Materials and methods

A field experiment was conducted during two crop years (1995–96 and 1996–97) at the Indian Agricultural Research Institute, New Delhi (28°38' N latitude; 77°11' E longitude). The crop year in India starts with the onset of the monsoon in July and ends in the June of the succeeding year. There are three crop growing seasons, namely, kharif (July–November) when rice is grown, rabi (November–April) when wheat is grown and summer (May–June) when a short duration legume like mungbean is grown. The present study was started with rice followed by wheat and mungbean in each crop year and thus six crops were grown in the two years of study.

The soil of the experimental field was a sandy clay loam Fluvent having 51.1% sand, 23.7% silt and 25.2% clay, pH 8.2 (1:2.5 soil to water ratio), 0.55% organic C, 319 kg ha^{-1} alkaline permanganate-hydrolysable N, 19.0 kg ha^{-1} 0.5 M $NaHCO_3$-extractable P and 285 kg ha^{-1} 1 N NH_4OAc-exchangeable K, determined using the procedures described by Prasad (1998). A rice–wheat cropping system had been practised in this field for the last 10 years.

The experiment was conducted in a randomized block design having four replications. The treatments consisted of N, NP, NK, NPK, NPK + Zn and NPK + FYM. The nitrogen was applied at 120 kg ha^{-1}, P at 26 kg ha^{-1}, K at 33 kg ha^{-1}, Zn at 5 kg ha^{-1} and FYM at 10 t ha^{-1}. These treatments were applied to both rice and wheat in both the years and mungbean was grown as a residual crop.

Rice (variety Pusa Basmati 1) was transplanted in the first week of July after the plots were bunded, flooded with water and puddled. Two to three 25–30-day-old seedlings of rice were transplanted per hill at a spacing of 20 cm × 10 cm. Nitrogen as urea was applied in 2 split doses, the first half 10 days after transplanting and the rest at the panicle initiation stage, and P as diammonium phosphate, K as muriate of potash, Zn as zinc sulphate heptahydrate and FYM were applied at the final puddling. The rice was harvested during the first week of November.

After the rice harvest, the field was irrigated and when the soil came to condition, it was disked twice and levelled. Before the second disking, half the dose of N as urea, the full dose of P as diammonium phosphate, K as muriate of potash and Zn as zinc sulphate heptahydrate and FYM were broadcast and incorporated into the soil. Wheat variety HD 2329 was sown in the third week of November and harvested in the second week of April.

Mungbean was sown in the last week of April after field preparation, and mature pods were picked in the last week of June. The green plants were incorporated into the soil by ploughing, followed by flooding and puddling in preparation for rice transplanting.

Results and discussion

Grain yield

The application of NP significantly increased the grain yields of rice, wheat and mungbean compared to N alone in 1995–96, whereas in 1996–97 this increase by P failed to reach the level of significance (Table 1). The application of NK significantly increased the grain yields of rice and wheat compared to N alone in the first year but not in the second year. The application of NPK significantly increased the grain yields of rice and wheat in both the years compared to N alone but not compared to NP or NK, indicating that the significant increase in the grain yield of rice and wheat by NPK over N alone was due to the combined effect of P and K. The application of Zn only had a significant effect on the grain yield of rice and mungbean in 1995–96, increasing the total productivity of the rice–wheat–mungbean cropping system by 0.6 t ha^{-1} compared to NPK. Farmyard manure, on the other hand, made a significant contribution to the rice yield only in 1995–96 and to the wheat yield in both the years, increasing the total productivity of the rice–wheat–mungbean cropping system by 0.6 t ha^{-1} in 1995–96 and 0.3 t ha^{-1} in 1996–97. Mishra and Prasad (2000) also reported the beneficial effect of FYM application along with inorganic nutrients in a rice–wheat cropping system.

Straw yield

The application of NP or NK had no significant effect on the straw yield of rice, wheat and mungbean as opposed to N alone in either of the years, whereas the application of NPK significantly increased the straw yield of the system in 1996–97, but not in 1995–96 (Table 1). The application of Zn had no significant effect on the straw yield of individual crops or the system as a whole in either year, whereas FYM significantly increased the total straw yield of the system compared to NPK in 1995–96 and to N alone in both the years.

Nutrient uptake

The rice–wheat–mungbean cropping system heavily depleted the soil, removing 220–280 kg N, 30–50 kg P and 210–270 kg K ha^{-1} year^{-1}. In both the years, the application of NP or NK resulted in a significant increase in the N, P and K uptake of rice compared with N alone (Table 2), while the application of NPK resulted in significantly better N, P and K uptake by rice than the N, NP or NK combinations. A further increase was recorded when NPK was combined with FYM. These results thus indicate that the effect of different nutrient combinations on the nutrient uptake of rice was greater than on the grain and straw yields of rice, due to their favourable effect on the nutrient concentration in the grain and straw of the crops.

Table 1

Effect of different nutrient combinations on the productivity (t ha^{-1}) of a rice–wheat–mungbean cropping system

Nutrient combinations	1995–96				1996–97			
	Rice	Wheat	Mung	Total	Rice	Wheat	Mung	Total
				Grain				
N	4.1	3.8	0.4	8.3	4.6	4.3	0.2	9.1
NP	4.3	4.1	0.5	8.9	4.8	4.5	0.3	9.6
NK	4.3	4.1	0.4	8.8	4.8	4.3	0.3	9.4
NPK	4.3	4.3	0.5	9.1	4.9	4.8	0.3	10.0
NPK + Zn	4.6	4.5	0.6	9.7	4.9	4.9	0.3	10.1
NPK + FYM	4.6	4.6	0.5	9.7	4.9	5.1	0.3	10.3
LSD (P=0.05)	0.19	0.24	0.10	–	0.41	0.70	0.24	–
				Straw				
N	5.7	5.4	3.4	14.5	5.6	5.5	2.4	13.5
NP	5.8	5.7	3.6	15.1	6.3	6.1	2.6	15.0
NK	5.7	5.7	3.4	14.8	6.0	5.9	2.5	14.4
NPK	5.8	5.7	3.6	15.1	7.3	6.3	3.1	16.7
NPK + Zn	5.9	5.8	3.5	15.2	7.2	6.1	3.1	16.4
NPK + FYM	6.0	5.8	4.0	15.8	7.3	6.3	3.3	16.9
LSD (P=0.05)	0.15	0.35	0.41	–	0.11	0.45	0.25	–

The N uptake of wheat was significantly increased by NP and NK compared to N alone in 1995–96 and by NPK compared to N, NP and NK in both the years (Table 2). The application of NPK + FYM only significantly increased the N uptake of wheat compared to NPK in 1995–96. Thus, the N uptake of wheat followed a pattern similar to that observed in the case of the grain and straw yields of wheat.

The application of NP and NK had no significant effect on the P uptake of wheat compared to N alone in either year, and the application of NPK only led to significantly greater P uptake than N and NK in 1995–96 (Table 2). Similarly, the application of NPK + FYM significantly increased the P uptake of wheat compared to N, NP and NK combinations in both the years.

The application of NP, NK and NPK had no significant effect on the K uptake by wheat, which increased only when FYM was applied along with NPK (Table 2). The beneficial effect of the combined use of organic and inorganic sources of nutrients on the NPK uptake may be attributed to the balanced nutrition of the crop plants (Meelu and Rekhi, 1981) and to the improvement in the physical and biological properties of the soil (Minhus and Sood, 1994).

Compared to N alone, the application of NP significantly increased the P and K uptake by mungbean in 1996–97, whereas NK had no significant effect on the N, P and K uptake of mungbean in either year of the study (Table 2). The application of NPK significantly increased the N, P and K uptake of mungbean compared with N, NP and NK in both the years. The application of Zn had no significant effect on the N, P and K uptake of mungbean, whereas the application of FYM significantly increased the N uptake in both the years and the P and K uptake in 1996–97.

Soil fertility

The organic C content of the soil increased significantly only when a combination of NPK + FYM was applied (Table 3). With this combination, the organic C content was maintained between 0.62 and 0.69% as against 0.49–0.57% with various combinations of mineral nutrients. Thus, a 21–26% increase in organic C content was observed after FYM application. An increase in the organic C content of the soil due to FYM application was also reported by Gaur (1984) and Nambiar (1994). It is also interesting to note that the organic C content decreased from one season to the other during the course of two years when only mineral nutrients were applied, whereas with NPK + FYM the organic C content increased from 0.62 to 0.69% during the study period.

The application of different combinations of N, P and K had no significant effect on the available N content of the soil, whereas the application of NPK + FYM significantly increased it compared with all combinations of N, P and K (Table 3). The significant effect of organic manures on the available N content was also observed by Nambiar (1994) and Balgopalan et al. (1994).

Table 2
Effect of different nutrient combinations on the NPK uptake (kg ha^{-1}) by a rice–wheat–mungbean cropping system

Nutrient combinations	1995–96				1996–97			
	Rice	Wheat	Mung	Total	Rice	Wheat	Mung	Total
				Nitrogen				
N	82.4	81.6	64.6	228.6	90.8	90.7	39.6	221.1
NP	87.3	89.7	66.8	243.8	98.8	98.1	42.9	239.8
NK	87.4	86.4	71.6	245.4	94.5	90.9	42.0	227.4
NPK	92.5	94.8	78.9	266.2	105.4	105.3	49.2	259.9
NPK + Zn	96.5	97.4	77.6	271.5	108.0	105.5	50.3	263.8
NPK + FYM	100.8	101.2	84.5	286.5	114.4	112.6	55.0	282.0
LSD (P=0.05)	1.37	0.48	8.60	–	4.59	14.7	4.00	–
				Phosphorus				
N	13.2	13.8	5.0	32.0	15.2	15.3	3.0	33.5
NP	15.5	15.3	5.5	36.3	16.3	17.3	3.7	36.3
NK	15.7	14.4	5.9	36.0	15.5	15.0	3.4	33.9
NPK	17.0	16.3	6.7	40.0	17.6	18.0	4.4	40.0
NPK + Zn	17.3	17.0	6.8	41.1	18.0	18.9	4.5	41.4
NPK + FYM	17.6	18.1	7.3	43.0	20.0	21.1	5.0	46.1
LSD (P=0.05)	0.64	1.73	0.98	–	1.49	3.27	0.53	–
				Potassium				
N	94.1	98.4	18.1	210.6	98.0	109.6	10.8	218.4
NP	97.5	103.3	19.2	220.0	108.6	109.3	12.2	230.1
NK	97.7	101.4	20.5	219.6	98.6	102.4	11.7	212.7
NPK	101.1	104.6	23.2	228.9	116.8	113.8	15.1	245.7
NPK + Zn	102.0	106.5	23.2	231.7	117.5	111.4	15.3	244.2
NPK + FYM	107.3	110.1	24.8	242.2	123.5	727.8	17.0	268.3
LSD (P=0.05)	2.29	6.46	3.36	–	5.01	16.4	1.37	–

The available P content was significantly greater in plots which received P fertilizers. It was further increased by the application of FYM along with NPK (Table 3). This combination (NPK + FYM) maintained the available P level between 25 and 34 kg ha^{-1} as against 18–27 kg ha^{-1} without P application and 21–32 kg ha^{-1} with P application. This might be due to the additional P available from the FYM and to the favourable effect of FYM on the availability of native P (Bhardwaj and Tandon, 1981; Singh, 1994).

The application of K along with N (NK) significantly increased the available K content of the soil by 3–14 kg ha^{-1} during the first year and by 14–18 kg ha^{-1} during the second year compared to N alone. The application of K along with N and P (NPK) was more effective, increasing the available K content of the soil by 3–16 kg ha^{-1} during the first year and by 16–20 kg ha^{-1} during the second year compared to N alone (Table 3). The available K content of the soil was further increased significantly by the application of FYM along with NPK. This might be due to the additional K available from FYM. Bhardwaj et al. (1994) also reported a significant increase in the level of soil K after FYM application.

Table 3
Effect of different nutrient combinations on the organic C, available N, available P and available K content of the soil

Nutrient combinations	1995–96			1996–97		
	After rice	After wheat	After mung	After rice	After wheat	After mung
			Organic C (%)			
N	0.54	0.49	0.53	0.52	0.49	0.52
NP	0.55	0.51	0.54	0.53	0.53	0.54
NK	0.53	0.50	0.54	0.55	0.53	0.53
NPK	0.56	0.52	0.55	0.56	0.54	0.56
NPK + Zn	0.57	0.53	0.55	0.55	0.55	0.57
NPK + FYM	0.63	0.62	0.65	0.67	0.66	0.69
LSD (P=0.05)	0.03	0.02	0.03	0.04	0.03	0.05
			Available N (kg ha^{-1})			
N	313.5	314.4	317.4	326.7	326.3	328.5
NP	315.5	315.0	319.4	328.4	327.9	329.0
NK	314.4	313.4	319.2	327.7	329.0	329.5
NPK	319.0	318.7	321.1	330.0	330.0	333.3
NPK + Zn	319.4	320.4	324.2	331.3	331.3	334.8
NPK + FYM	325.0	326.0	329.4	337.7	340.6	344.0
LSD (P=0.05)	3.6	5.1	6.7	5.1	6.9	8.0
			AvailableP(kgha^{-1})			
N	17.9	18.8	25.0	24.3	20.9	23.7
NP	20.5	22.8	25.5	29.6	28.5	27.8
NK	17.9	19.8	25.1	26.7	24.2	26.6
NPK	21.9	23.3	28.1	32.4	28.4	28.2
NPK + Zn	22.0	25.0	27.9	32.4	28.7	28.7
NPK + FYM	25.2	26.6	28.6	34.0	32.0	32.9
LSD (P=0.05)	1.8	4.2	2.6	5.0	4.1	4.8
			AvailableK(kgha^{-1})			
N	280.2	271.2	276.1	272.4	264.0	276.1
NP	281.1	272.6	274.7	273.1	264.5	277.2
NK	283.1	285.3	287.3	289.3	282.2	290.2
NPK	283.5	287.2	288.8	290.3	283.8	291.7
NPK + Zn	284.1	287.5	289.4	291.4	284.4	294.9
NPK + FYM	287.1	291.3	295.3	298.2	297.4	306.7
LSD (P=0.05)	3.8	2.9	4.1	6.6	9.8	9.3

The present study indicates that integrated nutrient management involving NPK fertilizers and FYM significantly increased both the productivity and soil fertility of a highly intensive rice–wheat–mungbean cropping system, thus holding out promise for the sustainability of the cropping system.

References

Balgopalan, M., Jose, A. I., Sheetalakshmi, K. K. (1994): Integrated management of organic matter, nitrogen, phosphorus and potassium for rice. *Adv. Plant Sci.*, **7**, 91–95.

Bhardwaj, B., Venkatesh, S., Omanwar, P. K. (1994): Long-term effect of continuous rotational cropping and fertilization on crop yields and soil properties. II. Effect on EC, pH, organic matter and available nutrients of soil. *J. Indian Soc. Soil Sci.*, **42**, 387–392.

Bhardwaj, R. B. L., Tandon, H. L. S. (1981): Fertilizer use research in some wheat based cropping system. *Fert. News,* **26,** 23–32.

Fujisaka, S., Harrington, L., Hobbs, P. (1994): Rice–wheat in South Asia: System and long term practices established through diagnosis research. *Agric. Systems,* **46**, 170–187.

Gaur, A. C. (1984): Response of rice to organic matter: The Indian experience. In: *Organic Matter and Rice*. IRRI, Manila, Philippines, pp. 503–514.

Meelu, O. P., Rekhi, R. S. (1981): Mung straw management and nitrogen economy in rice culture. *Int. Rice Res. Newsletter,* **6,** 21.

Minhus, R. S., Sood, A. (1994): Effect of inorganics and organics on the yield and nutrient uptake by three crops in rotation on an acid alfisol. *J. Indian Soc. Soil Sci.,* **42,** 257–260.

Mishra, B. N., Prasad, R. (2000): Integrated nutrient management for sustained production in a rice-wheat cropping system. *Acta Agron. Hung.*, **48**, 257–262.

Nambiar, K. K. M. (1994): *Soil Fertility and Crop Productivity under Long Term Fertilizer use in India*. Indian Council of Agricultural Research, New Delhi.

Prasad, R. (1998): *A Practical Manual of Soil Fertility*. Division of Agronomy, Indian Agricultural Research Institute, New Delhi.

Prasad, R., Mishra, B. N. (2001): Effect of addition of organic residue, farmyard manure and fertilizer nitrogen on soil fertility in rice-wheat cropping system. *Arch. Acker. Pfl. Boden.*, **46**, 455–463.

Sharma, S. N., Prasad, R. (1999): Effect of *Sesbania* green manuring and mungbean residue incorporation on productivity and nitrogen uptake of a rice-wheat cropping system. *Bioresource Tech.*, **67**, 171–175.

Sharma, S. N., Prasad, R., Singh, S. (1995): Role of mungbean residues and *Sesbania aculeata* green manure in the nitrogen economy of rice-wheat cropping system. *Plant Soil*, **172**, 123–129.

Singh, B., Singh, Y., Sekhon, G. S. (1995): Fertilizer nitrogen use efficiency and nitrate pollution of groundnut in developing countries. *J. Contaminant Hydro.*, **20,** 167–174.

Singh, Y. (1994): Integrated nutrient management in rice-wheat cropping system. National Symposium on "*Integrated Input Management for Efficient Crop Production*" held at Tamil Nadu Agricultural University, Coimbatore, India. Feb. 22–25, 1994.

Yadav, R. L. (1998): Factor productivity trends in rice-wheat cropping system under long-term use of chemical fertilizer. *Expt. Agric.*, **34**, 1–18.

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INFLUENCE OF VOLUNTEER DURUM WHEAT (*Triticum durum*) CULTIVARS AND DENSITY ON LENTILS (*Lens culinaris*)

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Greenhouse experiments were conducted for two consecutive seasons to investigate the influence of volunteer durum wheat cultivars and density on lentil growth and yield. It is speculated that interference may be severe whenever wheat and lentils are rotated in semi-arid regions. Lentil:durum wheat ratios of 1:0, 1:1, 1:2, 1:4 and 1:6 were evaluated utilizing two durum wheat cultivars, Hourani and ACSAD 65. The results indicated that wheat interference did not influence lentil growth for the first 80 days after crop emergence, but afterwards, the lentil height, straw and seed yield were affected by the presence of wheat. A 50% reduction in either lentil straw or seed yield was estimated from the interference of a single plant per pot. The semi-tall cultivar Hourani had more adverse effects on lentil growth and yield than the semi-dwarf cultivar ACSAD 65.

Key words: crop rotation, intercropping, interference, Jordan, Mediterranean, semi-arid environments, volunteer crops, WANA region.

Introduction

Volunteer crops emerging in subsequent crops are considered to be serious weeds for many reasons. When present at high densities, with uniform emergence and growth patterns, volunteer plants, which are adapted to the prevailing environmental conditions, interfere vigorously with the current crop and diminish the positive effects of crop rotation. Volunteer crops may increase the inoculum potential of necrotrophs, carry soil-borne pathogens through break crops, and act as carriers of crop pathogens, nematodes and insects (Chamberlin et al., 1992; Yarham and Gladders, 1993). Potatoes (*Solanum tuberosum* L.) (Boydston and Williams, 2003), cereals (Clarke, 1993), legumes (Knott, 1993), sugarbeet (*Beta vulgaris* L.) (Longden, 1993) and canola (*Brassica napus* L.) (Simard et al., 2002) are among the crops that have received special attention as volunteer crops.

The presence of volunteer crops is becoming of significant agro-ecological concern, given the large-scale use of herbicide-tolerant varieties in some areas (Anderson and Soper, 2003; Simard et al., 2002). The practice of reduced tillage was also associated with an increase in the negative impacts of volunteer crops (Derksen et al., 1993). A reduction in the tillage depth increased populations of perennial weeds and volunteer crops in Switzerland (Mayor and Maillard, 1995). When reviewing the impact of volunteer crops, Orson (1993) concluded that the control of volunteer crops requires as much attention as the control of weeds, and might involve extra cultivations and extra herbicide applications.

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In the WANA (West Asia and North Africa) region, the production of wheat (*Triticum aestivum* L. and *T. durum* L.) and barley (*Hordeum vulgare* L.) dominates dryland agriculture, which takes place under conditions of limited, variable or chronically deficient precipitation. These crops are grown in rotation with food legume crops, mainly chickpea and lentil (Pala et al., 1999). Mechanical wheat harvesting results in the natural shedding and shattering of grains, leaving numerous wheat kernels on the soil surface. Previous surveys in the UK indicated that wheat grain losses at harvest ranged from 2 to 6% and left approximately 240 to 700 kernels m^{-2} (Anderson and Soper, 2003). Wheat grain losses in developing countries are expected to be higher, since the maintenance and adjustment of harvest equipment is less precise.

Wheat is often considered to be less competitive than barley in temperate semi-arid and sub-humid climates, though Karim (2000) found wheat to be more competitive than barley or field bean (*Vicia faba* L.) in Pakistan. Lentil, on the other hand, was frequently described as the least competitive among a number of crops (Esser et al., 1999; Keatinge and Chapanian, 1991; Pandey et al., 1998). Lentil was less competitive than pea against volunteer oat (*Avena fatua* L.) and yellow mustard (*Sinapis alba* L.). Densities of 10 weeds m^{-2} significantly reduced lentil yields compared to weed-free lentil (Hornford and Drew, 1986). Weed interference is the main constraint of lentil production in Jordan (Snobar and Haddad, 1998).

Although the presence of volunteer wheat in lentil fields in the WANA region, including Jordan, is speculated to be high, the impact on lentil production is unknown and underestimated. Therefore, the objective of this research was to test the competitive effects of volunteer wheat cultivars and density on lentil growth, straw and seed yield.

Materials and methods

Greenhouse experiments were conducted for two successive seasons on the Jordan University of Science and Technology campus to investigate the effects of volunteer durum wheat interference on lentils. Seeds of lentil cv. Jordan 1, a cultivar widely planted in Jordan, were planted with seeds of either Hourani or ACSAD 65 durum wheat in pots with a 0.071 m^{-2} surface area containing 3 L of peat moss. Hourani is a local semi-tall (90–110 cm) durum wheat cultivar that is preferred by producers for its adaptability and tolerance to environmental fluctuations. ACSAD 65 is an improved semi-dwarf (60–75 cm) durum cultivar that is adapted to many production regions of Jordan. Farmers utilize both durum wheat cultivars interchangeably within and across production areas.

The crops were planted at lentil:wheat densities of 1:0, 1:1, 1:2, 1:4 and 1:6 plants per pot in experiments established in December 1998 and 1999 by planting seeds of both crops and thinning to the proper ratios 3 days after emergence. The lentil plants were placed in the centre of the pot whenever applicable and were surrounded by wheat plants. The pots were arranged in a randomized complete block design with all treatments replicated three times. Because the experiments included destructive measurements, three sets of replicated treatments were included. The first and second sets were terminated at 40 and 80 days after emergence (DAE), respectively, whereas the third set was harvested after seed maturity but before lentil seed shattering (150 DAE). Adequate moisture was maintained throughout the experiments and 150 ml of Hoagland-Arnon nutrient solution was applied three times (Hoagland and Arnon, 1950). Greenhouse temperatures were 28/16°C day/night.

Plant height, measured from soil level to the uppermost leaf tip, and the number of emerging leaves were recorded weekly until 119 DAE. Fresh and oven-dry (48 h at 72°C) shoot weights of lentils and wheat were recorded 40 and 80 days after planting. At maturity, oven-dry (48 h at 72° C) seed and straw weights per pot were recorded for both crops.

Analysis of variance was performed using the general linear model procedure (SAS, 1989) to detect significant year and treatment effects. Since neither the year nor the year by treatment effects were significant, the data were pooled across years. Linear regression analysis was performed on the raw data taking lentil and wheat height, leaf number, wheat straw yield and wheat grain yield as dependent variables, and wheat density as the independent variable. Because the lentil straw and seed yields had an asymptotic relationship with wheat density, non-linear regression analysis was performed on the raw data of these two variables (SAS, 1989). The three-parameter exponential function described by Chism et al. (1992) was modified as follows:

$$Wt = B_0 + B_1 \times e\,(-B_2 \times D) \qquad [1]$$

where Wt was the dry weight of lentil straw or seed per plant, B_0 the lower asymptotic weight, B_1 the reduction in plant weight from the upper to the lower asymptote, B_2 the rate at which Wt achieved the lower weight asymptote, and D the wheat density. Wt was expressed as g plant^{-1} and B_2 as the inverse of wheat density. Pseudo-R^2 estimates were used to assess the goodness of fit, and pair-wise comparisons between coefficients were performed as described by Chism et al. (1992).

Results and discussion

Lentil height was not affected by the presence of wheat plants for the first 91 DAE (data not presented). Apparently, resources were not limited at that stage. However, when height measurements were recorded 98 or 112 DAE, the lentil height was negatively affected by wheat density and the plants were shorter when grown with Hourani than with ACSAD 65. The height-decline rate (slope) was not affected by the wheat cultivars (Table 1). The number of lentil leaves was not affected by wheat interference, indicating that this variable was not affected by competition (data not presented).

The fresh and dry lentil weights recorded 40 or 80 DAE were not affected by wheat cultivars or wheat density, again indicating less crucial resource limitations (data not presented). These results disagree with reports describing the critical period of weed interference, dominated by broad-leaved weeds, to be between 49 and 56 days after crop emergence (Curran et al., 1987; Singh et al., 1996). In later stages the straw and seed yield responses to wheat interference were curvilinear, and were described excellently (R^2>0.90) by the three-parameter exponential function (Figs. 1 and 2).

Table 1

Linear regression coefficients for the response of lentil height to interference by durum wheat cultivars 98 and 112 days after emergence

Days after emergence	Crop mixture	a	b
98	Lentil:Hourani	53.99 (±3.10[a])	–2.01 (±0.92)
	Lentil:ACSAD 65	59.50 (±2.10)	–2.72 (±0.88)
112	Lentil:Hourani	61.56 (±3.60)	–2.01 (±1.10)
	Lentil:ACSAD 65	70.06 (±4.42)	–3.42 (±1.31)

[a]Values between parentheses are SE; General equation: lentil height (cm) = a + b (wheat density)

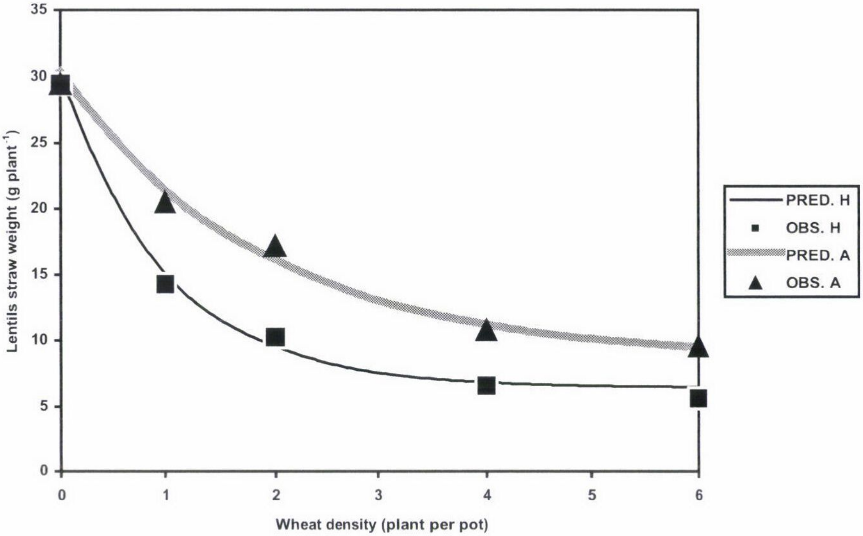

Fig. 1. Response of lentil straw yield to wheat density and cultivar interference. Regression equations: for Hourani $Wt_{straw} = 6.44 + 23.94 * e (-1.02 * \text{Wheat density})$ ($R^2 = 0.95$) and for ACSAD 65 $Wt_{straw} = 8.61 + 21.8 * e (-0.53 * \text{Wheat density})$ ($R^2 = 0.92$). Abbreviations: PRED. = Predicted, OBS. = Observed, A = ACSAD 65 and H = Hourani

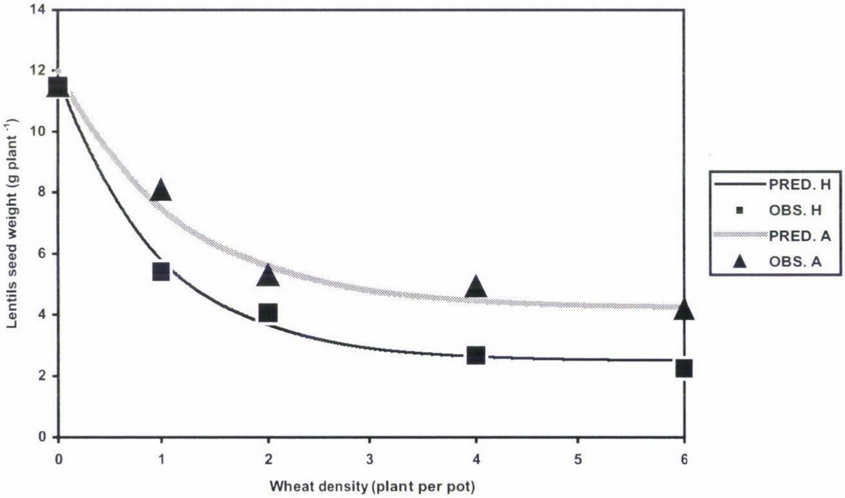

Fig. 2. Response of lentil seed yield to wheat density and cultivar interference. Regression equations: for Hourani $Wt_{seed} = 2.51 + 9.34 * e (-1.04 * \text{Wheat density})$ ($R^2 = 0.90$) and for ACSAD 65 $Wt_{seed} = 4.20 + 7.78 * e (-0.83 * \text{Wheat density})$ ($R^2 = 0.91$). Abbreviations: PRED. = Predicted, OBS. = Observed, A = ACSAD 65, H = Hourani

The non-linear regression coefficients for lentil straw yield indicated that the lower asymptote (B_o) and the rate of decline (B_2) were lower in the lentil:Hourani mixture than in the lentil:ACSAD 65 mixture (Table 2). Straw yield reductions from the presence of a single wheat plant per pot (or alternatively 14 wheat plants m^{-2} of soil surface) were predicted to be 50 and 30% of the wheat-free lentil yield for the Hourani and ACSAD 65 mixtures, respectively (Fig. 1). At a wheat density of 6 plants per pot, the straw yield reductions from Hourani and ACSAD 65 interference were estimated to be 79 and 68%, respectively, compared to lentils grown alone. The lentil seed yield response to durum wheat interference was also characterized by severe losses (Fig. 2), with similar values for the two cultivars; only the lower asymptotes (B_o) were different for the two regression equations (Table 2). Hourani interference was estimated to reduce the lentil seed yield by 51 and 79% at densities of 1 and 6 plants per pot, respectively, compared to the wheat-free lentil seed yield, while ACSAD 65 interference reduced the lentil seed yield by 37 and 65% at densities of 1 and 6 plants per pot, respectively (Table 2). Mishra et al. (1997) described lentil yield reductions from *Vicia sativa* L. competition to range from 21% at 30 plants m^{-2} to 37% at 180 plants m^{-2}. The present results reveal more severe losses in lentil yield from wheat interference and are comparable with those of Carr et al. (1995), who found that wheat-lentil intercropping was inappropriate in cool semi-arid regions due to low lentil yields.

To obtain a better understanding of the influence of wheat density and cultivar on lentils, the wheat height and leaf number were measured weekly. The wheat leaf number was not affected by either wheat density or cultivar throughout the season (data not presented). Height differences between the two wheat cultivars were not detected for the first 70 DAE. Measurements recorded at 77 DAE revealed that ACSAD 65 was taller than Hourani, which is related to the early maturing growth habit of ACSAD 65 (Table 3). Later on, heights were either equivalent (84 DAE) or Hourani was taller (91 DAE and after). The influence of increased wheat density on the height of the two varieties was similar at 77 DAE, after which the plant height of Hourani had a significantly steeper slope, indicating that density influenced Hourani more than ACSAD 65 (Table 3).

Table 2

Non-linear regression coefficients for the response of lentil straw and seed yield to interference by durum wheat cultivars

Crop mixture	Lentil straw yield			Lentil seed yield		
	B_o	B_1	B_2	B_o	B_1	B_2
Lentil:Hourani	6.44 (±1.62[a])	23.94 (± 2.16)	1.02 (± 0.27)	2.50 (±0.75)	9.34 (±1.00)	1.04 (±0.33)
Lentil:ACSAD 65	8.61 (±1.25)	21.80 (±3.47)	0.53 (±0.23)	4.20 (±1.00)	7.78 (±1.30)	0.86 (±0.40)

General equation: $Wt_{(\text{straw or seed yield})} = B_o + B_1 \times e\,(-B_2 \times \text{Wheat density})$; [a]Values between parentheses are asymptotic SE

Table 3

Linear regression coefficients for the response of wheat height to wheat cultivars and density in lentil:wheat mixtures at 77, 84, 91, 98 and 112 days after emergence

Days after emergence	Crop mixture	a	b
77	Lentil:Hourani	68.12 (±3.64[a])	–2.49 (±0.97)
	Lentil:ACSAD 65	77.67 (±1.51)	–2.32 (±0.40)
84	Lentil:Hourani	80.85 (±4.50)	–3.86 (±1.19)
	Lentil:ACSAD 65	78.95 (±0.92)	–2.21 (±0.24)
91	Lentil:Hourani	89.5 (±3.15)	–4.11 (±0.85)
	Lentil:ACSAD 65	83.33 (±1.85)	–2.30 (±0.49)
98	Lentil:Hourani	102.88 (±4.60)	–3.01 (±0.95)
	Lentil:ACSAD 65	83.38 (±1.50)	–1.81 (±0.40)
112	Lentil:Hourani	122.96 (±4.18)	–2.55 (±1.11)
	Lentil:ACSAD 65	83.13 (±1.27)	–0.85 (±0.34)

General equation: wheat height (cm) = a + b (wheat density).[a]Values between parentheses are SE

The substantial straw and seed yield reductions recorded in this experiment emphasize the importance of controlling volunteer wheat in lentil fields. Given the fact that lentil straw is among the most valuable forages in semi-arid regions, the reported reductions in straw yield should also be taken into consideration. The lack of reduction in lentil height and biomass in the first 80 DAE might imply the availability of resources or the ability of lentil plants to successfully compete with volunteer wheat for an extended period of time. However, the results indicate that lentil fields should be kept free of weeds and volunteer wheat for more than three months after crop emergence to avoid severe yield losses. The wheat cultivar Hourani was characterized by taller plants and greater straw production, resulting in greater shading and higher demands for moisture and nutrients, which could explain the more severe reductions in the height, straw and seed yield of lentils in the presence of Hourani compared to ACSAD 65 interference. ACSAD 65 is known to outyield Hourani in grain yield and to withstand higher planting densities in well-watered and fertilized farming systems. The lack of difference between the two wheat cultivars in grain yield in the present experiment is indicative of the greater competitive influence of lentil plants on ACSAD 65 compared to Hourani and partially explains the lower effect of ACSAD 65 on the lentil straw and seed yields. These results will alert farm managers and producers to the impact of volunteer wheat interference in lentil, a case that is rarely considered a problematic infestation.

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References

Anderson, R. L., Soper, G. (2003): Review of volunteer wheat (*Triticum aestivum*) seedling emergence and seed longevity in soil. *Weed Technol.*, **17**, 620–626.

Boydston, R. A., Williams II, M. M. (2003): Effect of soil fumigation on volunteer potato (*Solanum tuberosum*) tuber viability. *Weed Technol.*, **17**, 352–357.

Carr, P. M., Gardner, J. C., Schatz, B. G., Zwinger, S. W., Guldan, S. J. (1995): Grain yield and weed biomass of a wheat-lentil intercrop. *Agronomy J.*, **87**, 574–579.

Chamberlin, J. R., Todd, J. W., Beshear, R. J., Culbreath, A. K., Demski, J. W. (1992): Over wintering hosts and wing form of thrips, *Frankliniella* spp., in Georgia: implications for management of spotted wilt disease. *Environmental Entomology*, **21**, 121–128.

Chism, W. J., Birch, J. B., Bingham, S. W. (1992): Nonlinear regression for analyzing growth stage and quinclorac interactions. *Weed Technol.*, **6**, 898–903.

Clarke, J. H. (1993): Set-aside: a weeder and seeder of volunteer crops. *Aspects of Applied Bio.*, **35**, 215–222.

Curran, W. S., Morrow, L. A., Whitesides, R. E. (1987): Lentil (*Lens culinaris*) yield as influenced by duration of wild oat (*Avena fatua*) interference. *Weed Sci.*, **35**, 669–672.

Derksen, D. A., Lafond, G. P., Thomas, A. G., Loeppky, H. A., Swanton, C. J. (1993): Impact of agronomic practices on weed communities: tillage systems. *Weed Sci.*, **41**, 409–417.

Esser, A. D., Brown, J., Davis, J. B. (1999): Weed competition of yellow mustard, canola, pea and lentil. *Cruciferae Newsletter*, **21**, 145–146.

Hoagland, D. R., Arnon, D. I. (1950): The water culture method of growing plants without soil. *Berkeley: California Agricultural Experiment Station Circular 347.* 32 p.

Hornford, R. G., Drew, B. N. (1986): Yield reductions in field peas and lentils resulting from volunteer crop competition. *Proc. 32 Can. Pest Management Soc.*, Charlottetown, P.E.I., Canada. pp. 50–56.

Karim, S. M. R. (2000): Competitive ability of volunteer crops grown as weeds. *Pakistan J. Agri. Res.*, **16**, 142–146.

Keatinge, J. D. H., Chapanian, N. (1991): The effect of improved management on the yield and nitrogen content of legume hay/barley crop rotations in West Asia. *J. Agro. and Crop Sci.*, **167**, 61–69.

Knott, C. M. (1993): Volunteer crops in legumes for processing. *Aspects of Applied Biol.*, **35**, 207–213.

Longden, P. C. (1993): Weed beet: a review. *Aspects of Applied Biol.*, **35**, 185–194.

Mayor, J. P., Maillard, A. (1995): Results from an over-20-years-old ploughless tillage experiment at Changis. IV: Seed bank and weed control. *Revue Suisse d' Agriculture*, **27**, 229–236.

Mishra, J. S., Singh, V. P., Bhan, V. M. (1997): Effect of interference by common vetch (*Vicia sativa*) on yield and yield components of lentil (*Lens culinaris*). *Indian J. Agri. Sci.*, **67**, 320–321.

Orson, J. H. (1993): The penalties of volunteer crops as weeds. *Aspects of Applied Bio.*, **35**, 1–8.

Pala, M., van Duivenbooden, N., Studer, C., Bielders, C. L. (1999): Cropping systems and crop complementarity in dryland agriculture. *Proceedings of Efficient Soil Water Use Workshops.* International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India. pp. 299–330.

Pandey, A. K., Kamta, P., Singh, P., Singh, R. D., Prasad, K., Singh, P. (1998): Comparative yield loss assessment and crop-weed association in major winter crops of mid hills of North West Himalayas. *Indian J. Weed Sci.*, **30**, 54–57.

SAS (1989): *SAS User's Guide: Statistics*. Version 6, 4th ed. Statistical Analysis Systems Institute, Cary, NC.

Simard, M. J., Légère, A., Pageau, D., Lajeunesse, J., Warwick, S. (2002): The frequency and persistence of volunteer canola (*Brassica napus*) in Québec cropping systems. *Weed Technol.*, **16**, 433–439.

Singh, M., Saxena, M. C., Abu-Irmaileh, B. E., Al-Thahabi, S. A., Hadad, N. I. (1996): Estimation of critical period of weed control. *Weed Sci.*, **44**, 273–283.

Snobar, B. A., Haddad, N. I. (1998): Evaluation of weed control methods in lentil (*Lens culinaris* Med.) in Jordan. *Dirasat*, **25**, 203–213.

Yarham, D. J., Gladders, P. (1993): Effect of volunteer plants on crop diseases. *Aspects of Applied Bio.*, **35**, 75–82.

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A COMPARISON OF CYTOPLASMIC AND CHEMICALLY-INDUCED MALE STERILITY SYSTEMS FOR HYBRID PERFORMANCE IN WHEAT (*Triticum aestivum* L.)

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Cytoplasmic (*Triticum timopheevii*-based) and chemically induced (CH9832- based) male sterility systems were compared for hybrid performance in wheat. A total of 40 genotypes including 10 CMS-based hybrids, 10 CHA-based hybrids, 10 B lines and 10 R lines were included in the experiment. Analysis of variance revealed significant differences between the genotypes for all the characters studied. Differences between the hybrids and their parents were significant for all the characters. There were also significant differences between the hybrids resulting from the two systems of sterility. This study of 10 comparable hybrids showed, on average, midparent heterosis of 30.2 and 7.3% for CMS- and CHA-derived hybrids, respectively. Generally, the CMS-based hybrids were superior to their CHA-based equivalents for grain yield performance. In spite of the incomplete fertility the higher grain yield in CMS-based hybrids was attributed to the profuse tillering and high thousand-grain weight. On the other hand, the CHA-based hybrids had lower yield performance due to the lower thousand-grain weight and tillering. The lower grain weight and tillering in these hybrids compared with their CMS-based equivalents might be due to the toxic effect of the CHA, which was carried over to the hybrid and affected vigour. As a whole the CMS system was found to be better than the CHA system (based on the particular CHA used in this study) for hybrid performance in wheat.

Key words: CHA, CMS, heterosis, hybrids, wheat

Abbreviations: CHA = chemical hybridizing agent; CMS = cytoplasmic male sterility; R = restorer lines

Introduction

Experts predict that the demand for wheat will increase by 40% in the next 20 years (Cukadar and Van Ginkel, 2001). Meeting this increased demand constitutes an enormous challenge, especially because yield levels appear to have reached a plateau in recent years. Therefore, to increase wheat yield levels and meet the growing world demand for wheat, breeders need to exploit new technologies to facilitate the development of hybrids. The development of hybrid varieties is presently undergoing a detailed re-examination as a wheat breeding strategy. Notwithstanding the development of a CMS-based hybrid seed production system in wheat in the 1960s, the hybrid approach became sidelined with the advent of semi-dwarf cultivars. The current interest in hybrid wheat is explained by (a) gains from the conventional approach tending to plateau, (b) the emergence of effective chemical hybridizing agents (CHAs), and (c) the availability of new germplasm.

Heterosis has been observed in many self-fertilizing species and has been the object of considerable study as a means of increasing the productivity of wheat (*T. aestivum*) and other cereals (Bailey et al., 1980). Observations of heterosis in wheat date back to 1919, when Freeman studied the date of the first head, and the height and leaf width in crosses involving a durum wheat and three common wheats (Briggle, 1963). Some of the early studies showed a high frequency of yield heterosis. In 54 such trials reviewed by Pickett (1993), 29 contained hybrids giving more than 30% heterosis over the better parent (heterobeltiosis) for grain yield. Moreover, in 10 of these trials, heterosis in excess of 50% was reported. However, a large number of trials before 1975 and some work even later were based on a small quantity of seed produced by hand pollination and evaluated in single rows or small plots with widely spaced plants. Thus, this information on heterosis has little relevance with regard to commercial exploitation. Lucken and Johnson (1989) reported that CHA-based spring wheat hybrids, tested for four years in wheat performance trials in Kansas, yielded a maximum standard heterosis of 12% in a particular year. In hybrid yield trials in the UK, Germany, Australia and the USA, the maximum benefits in grain yield over the best line varieties ranged from 0.6% to 16.6% (Pickett, 1998). Bruns and Peterson (1998) reported that hybrid wheat in the Great Plains of the USA showed a fundamental yield responsiveness and selection gain advantage over pure line varieties that could result in acceptance by producers. According to Peterson et al. (1997), compared with pure line cultivars, hybrid wheats have the potential for enhanced mean yield and greater yield response to favourable environmental conditions with similar deviations from the expected response. Recently, in China, a yield advantage of up to 30% was reported for wheat hybrids produced using CHAs (Cukadar and Van Ginkel, 2001). Heterosis may be expressed in the form of plant height, general plant vigour, maturity, number of spikes per plant, number of kernels per spike, kernel weight, total grain yield per plant, and several other plant characters (Briggle, 1963).

The first hybrid wheat variety produced using the male sterility restoration system was released in the USA in 1978 by De Kalb and Pioneer International, though the work proved a failure because of instability in performance, inconsistent yields, greater susceptibility to diseases and impure seed stocks, which were responsible for its withdrawal in 1979 (Tandon, 1995). Similarly, RH01 (Frandoc × Festin) was the first commercial hybrid extensively tested in

Italy (Borghi et al., 1988). According to Carver et al. (1987) hybrids in the soft and hard red winter wheat classes were produced in the USA using the cytoplasmic-nuclear system of pollination control as well as an alternative system based on chemical pollen suppressants. A number of wheat hybrids that were developed through CHA have been submitted for commercial registration in several European countries and have entered the marketplace in both Europe and the United States (Cisar and Cooper, 2002). The Maharashtra Hybrid Seed Company (MAHYCO), in collaboration with Monsanto of the USA, is the only company that has commercialized hybrid wheat in India. Two CMS-based hybrid cultivars: Pratham 7070 and Pratham 7050, have been released recently for the Eastern, Central and Peninsular Zones of India.

Johnson (1977) suggested that in wheat-producing areas where the grain yield is less than 30 q/ha, the yield advantage required to compensate the extra cost of hybrid seed might be beyond the possibilities of current plant breeding. In contrast, where the grain yield is expected to be approximately 60–80 q/ha, the future for hybrids is much more favourable. The present experiment was carried out to compare the cytoplasmic and chemically-induced male sterility systems for hybrid performance in wheat.

Materials and methods

The experiment was conducted at the experimental farm of the Department of Plant Breeding, Punjab Agricultural University, Ludhiana (34°5'N, 75°48'E), India during the crop season November 2001–April 2002 under irrigated conditions.

Plant material

The hybrid seed produced in the first part of the experiment, November 2000–April 2001 (Adugna et al., 2004), formed the basis of this experiment. The pedigrees of the parents used to make the hybrids are presented in Table 1. This trial consisted of 10 CMS (*Triticum timopheevii*-based) hybrids, 10 CHA (CH9832-based) hybrids derived from parents corresponding to those of the CMS hybrids, 10 maintainer (B) lines and 10 restorer (R) lines representing the male parental lines used for both the CHA- and CMS-based hybrids included in the experiment. The B lines were used as the female parents of the CHA-based hybrids evaluated in the experiment. This set of B lines was also the counterpart of the A lines, which were the parents of the CMS-based hybrids. The B lines were thus included in the trial as parental checks. The genotypes evaluated in the experiment are indicated in Table 1.

Design and management practices

The genotypes were sown in a randomized complete block design and replicated three times. The plot size consisted of 4 rows, each 2 m in length, with row-to-row spacing of 23 cm. A seed rate of 18.2 g/plot was used, corresponding to the commercial seed rate of 99 kg/ha. Other management practices were those normally recommended for the area. All the hybrids were naturally pollinated by the wind, supplemented with some hand shaking using long sticks.

Table 1
List of hybrids and their parents included in the experiment

Entry No.	Designation/ pedigree
CMS-based hybrids	
1	CMS HD2687 × [CMSA/2*PBW343//Rfx (HWL1422)/3/PBW343]
2	CMS Dacula/ Chagual// Cazo × [CMSA/2*HD2687//Rfx (HWL1422)]
3	CMS Chilero × [CMSA/2*Turaco/Chilero//Rfx (HWL1422)]
4	CMS Turaco × [CMSA/2*HD2687//Rfx (HWL1422)]
5	CMS Kauz/ Hevo × [CMSA/2*HD2687//Rfx (HWL1422)/3/HD2687]
6	CMS PBW448 × [CMSA/2*Turaco/Chilero//Rfx (HWL1422)]
7	CMS Kauz*2/ MNV//Kauz × [CMSA/2*Turaco/Chilero//Rfx (HWL1422)]
8	CMS Munia/ Kauz × [CMSA/2*Chilero//Rfx (HWL1422)/3/Chilero]
9	CMS TJB368.251/ Buc// V81608 × [Rfx (HWL1422)/W8020]
10	CMS Varona/ Cno// Kauz × [Rfx (HWL1422)/W8020]
CHA-based hybrids	
11	HD2687 × [CMSA/2*PBW343//Rfx (HWL1422)/3/PBW343]
12	Dacula/ Chagual// Cazo × [CMSA/2*HD2687//Rfx (HWL1422)]
13	Chilero × [CMSA/2*Turaco/Chilero//Rfx (HWL1422)]
14	Turaco × [CMSA/2*HD2687//Rfx (HWL1422)]
15	Kauz/ Hevo × [CMSA/2*HD2687//Rfx (HWL1422)/3/HD2687]
16	PBW448 × [CMSA/2*Turaco/Chilero//Rfx (HWL1422)]
17	Kauz*2/ MNV//Kauz × [CMSA/2*Turaco/Chilero//Rfx (HWL1422)]
18	Munia/ Kauz × [CMSA/2*Chilero//Rfx (HWL1422)/3/Chilero]
19	TJB368.251/ Buc// V81608 x [Rfx (HWL1422)/W8020]
20	Varona/ Cno// Kauz × [Rfx (HWL1422)/W8020]
B lines	
21	HD2687
22	Dacula/ Chagual// Cazo
23	Chilero
24	Turaco
25	Kauz/ Hevo
26	PBW448
27	Kauz*2/ MNV//Kauz
28	Munia/ Kauz
29	TJB368.251/ Buc// V81608
30	Varona/ Cno// Kauz
R lines	
31	[CMSA/2*PBW343//Rfx (HWL1422)/3/PBW343]
32	[CMSA/2*HD2687//Rfx (HWL1422)]
33	[CMSA/2*Turaco/Chilero//Rfx (HWL1422)]
34	[CMSA/2*HD2687//Rfx (HWL1422)]
35	[CMSA/2*HD2687//Rfx (HWL1422)/3/HD2687]
36	[CMSA/2*Turaco/Chilero//Rfx (HWL1422)]
37	[CMSA/2*Turaco/Chilero//Rfx (HWL1422)]
38	[CMSA/2*Chilero//Rfx (HWL1422)/3/Chilero]
39	[Rfx (HWL1422)/W8020]
40	[Rfx (HWL1422)/W8020]

Observations and data analysis

Observations on days to ear emergence, plant height, number of grains per spike and thousand-grain weight were recorded following the same procedure as in Adugna et al. (2004). Additional data were recorded for tillers per metre (estimated from the average of two observations on the number of tillers in a one-metre row length), grain yield per plot and weight of ten ears (the weight of the grains from a sample of ten ears). The trial data were subjected to analysis of variance in a randomized block design for each character. A paired t-test was computed for means against each character of the hybrids derived from both systems of male sterility.

Results

Analysis of variance

Analysis of variance was computed for the trial comprising 40 entries grown in three replications. Differences between the genotypes for grain yield, days to 50% ear emergence, plant height, weight of ten ears, thousand-grain weight and number of grains per spike were significant (p=0.01). Analysis of variance for tillers per metre also showed a significant difference (p=0.05). The significance of grain yield, plant height and 1000-grain weight in the F_1 hybrids in comparison with the means of the parental cultivars was in conformation with that reported by Murai (1998) for *Ae. crassa*. However, the significant differences between the entries could be due to genotypic or sterilizing system differences. Partitioning of the genotype source of variation into hybrids (H), parents (P) and hybrids vs. parents (H/P) showed that differences between the hybrids were highly significant for all the characters except for weight of ten ears and tillers per metre (Table 2). Differences between the parents were also highly significant for all the characters. Moreover, the H/P source was highly significant, showing the presence of significant differences between the hybrids and their parents and the superiority of the hybrids over their parents for all the characters. The main interest was to compare the genetically equivalent hybrids derived from the two systems of male sterility. Further partitioning of the hybrid source of variation into CMS, CHA, and CMS vs. CHA (CMS/CHA) showed that CMS hybrids did not differ among themselves for any characters except days to 50% ear emergence. However, CHA-based hybrids were significantly different among themselves for days to 50% ear emergence, plant height, thousand-grain weight and number of grains per spike. The CMS/CHA source was highly significant for all the characters except for the weight of ten ears, indicating the presence of highly significant differences between the CMS and CHA hybrids and the superiority of the CMS-based hybrids to the CHA-based hybrids for all the characters except the weight of ten ears (Table 2). The paired t-test conducted for the CMS and CHA system means showed significant differences for all the characters. The differences in CMS/CHA-based hybrids could be expected if nucleo-cytoplasmic interactions affected plant characters positively or negatively (sterilizing the cytoplasm is known to confer a yield penalty), if restoration was incomplete leading to sterility in F_1, and if the toxic effect of CHA was carried over to the hybrids, thus affecting vigour (Adugna et al., 2004).

Table 2

Mean squares from the analysis of variance for *Triticum timopheevii* and chemical hybridizing agent (CH9832) based wheat hybrids for the various characters studied

Sources of variation	Degrees of freedom	Grain yield	Days to 50% ear emergence	Plant height	Weight of ten ears	Thousand-grain weight	Number of grains per spike	Tillers per metre
Replications	2	0.01	26.34^{**}	160.05^{**}	9.94	65.39^{**}	40.54	3333.84^{**}
Genotypes	39	0.07^{**}	59.49^{**}	158.09^{**}	22.93^{**}	54.35^{**}	172.36^{**}	803.06^{*}
Hybrids (H)	19	0.04^{**}	44.69^{**}	75.52^{**}	11.89	26.00^{**}	141.09^{**}	375.55
CMS	9	0.01	10.75^{*}	23.29	9.02	3.58	46.79	90.27
CHA	9	0.02	58.25^{**}	47.35^{**}	16.06	40.62^{**}	181.91^{**}	174.38
CMS/CHA	1	0.51^{**}	228.15^{**}	799.13^{**}	0.14	96.24^{**}	622.29^{**}	4753.67^{**}
Parents (P)	19	0.07^{**}	70.48^{**}	236.75^{**}	23.76^{**}	43.04^{**}	204.17^{**}	1123.79^{**}
H/P	1	0.59^{**}	132.35^{**}	226.95^{**}	216.75^{**}	807.46^{**}	162.16^{**}	2851.58^{**}
Error	78	0.01	4.43	12.91	9.09	8.01	25.68	436.43

*, ** Significant at the 0.05 and 0.01 levels of probability, respectively

Grain yield

Significant yield differences were observed between the CMS and CHA versions of the same cross combinations in 60% of the hybrids. The grand mean grain yield of the CMS-based hybrids was superior to that of the CHA-based hybrids and of the B and R parents (Table 3). All the CMS hybrids except Varona/Cno//Kauz × R_{20} consistently showed grain yields of over 1 kg/plot. By contrast, all the CHA-based hybrids gave mean grain yields of less than 1 kg/plot.

The hybrid giving the highest grain yield (CMS-based hybrid, Kauz/Hevo × R_8) resulted from the combination of parents which individually showed superior performance (Kauz/Hevo and R_8). However, there was no consistent evidence in this study indicating that high-yielding pure lines gave high-yielding hybrids. The increased grain yield in hybrids was in agreement with the reports given by a number of past authors. For example, Carver et al. (1987) found that hybrid cultivars generally performed at a higher yield level than pure line cultivars of hard red winter wheat. Despite the lack of complete fertility restoration, the higher grain yield obtained in CMS-based hybrids may probably be due to the increased grain weight and tillering. This was partially in agreement with the results of Murai (1998), who reported, based on a photoperiod-sensitive cytoplasmic male sterile form of *Ae. crassa*, that all F_1 hybrids showed heterosis for grain yield because of a higher spikelet number/ear and higher 1000-grain weight than their parental cultivars. Peterson et al. (1997) reported similar results. Despite the shrivelled seeds, the higher grain yield in the CHA-based hybrids compared with their parents was probably due to the dense sowing. Since the CHA-based hybrid seeds were shrivelled, the number of seeds sown could not be the same as for the CMS-based hybrids and the control, i.e. the number was higher.

Heterosis for grain yield

Generally, average/midparent heterosis was evident in both the CMS- and the CHA-based hybrids for grain yield. This was in the range of 12% to 63% in CMS-based hybrids and –1% to 54% in CHA-based hybrids (Table 3), though heterosis was below 10% in all but one CHA hybrid. Heterosis was significant for 8 of the 10 CMS-based hybrids. However, only one CHA-based hybrid showed significant midparent heterosis. The mean heterosis for CMS-based hybrids was 30.2%, whereas it was 7.3% for the CHA-based hybrids. Genotypic differences existed for heterosis in the test hybrids. Accordingly, only one hybrid gave the highest grain yield heterosis in both systems of sterility. Similarly, Barbosa et al. (1996) reported that midparent heterosis for grain yield ranged from –20 to 57% in CMS-based wheat hybrids.

Days to 50% ear emergence

ANOVA revealed significant differences between the genotypes for days to 50% ear emergence (Table 4). The overall mean comparison revealed that CMS hybrids were earlier than the CHA hybrids and the B and R parents (Table 5). However, the difference from the R parents was not significant. Six of the 10 CMS-based hybrids exhibited earlier ear emergence than the corresponding CHA-based hybrids, but the differences were not significant for the 10 CHA hybrids found to be earlier than their corresponding B lines. According to Borghi et al. (1988) the hybrids tended to be earlier, on average, than their female parents, which was in agreement with the results for CHA-based hybrids in this experiment.

Table 3

Effect of CMS and CHA systems on grain yield performance and expression of midparent heterosis (MPH) for grain yield (kg/plot) in wheat hybrids

Hybrids	Grain yield (kg/plot)				MPH (%)		
	CMS	CHA	B	R	MP	CMS	CHA
HD2687 × R_3	1.06^{*}	0.81	0.68	0.94	0.81	31^{*}	0^{ns}
Dacula/Chagual//Cazo × R_6	1.00	0.95	0.81	0.42	0.62	63^{*}	54^{*}
Chilero × R_9	1.01^{*}	0.81	0.80	0.83	0.82	24^{*}	-1^{ns}
Turaco × R_7	1.02^{*}	0.77	0.85	0.66	0.76	35^{*}	2^{ns}
Kauz/Hevo × R_8	1.21^{*}	0.99	1.01	0.97	0.99	22^{*}	0^{ns}
PBW448 × R_{11}	1.07^{*}	0.75	0.57	0.89	0.73	47^{*}	3^{ns}
Kauz*2/MNV//Kauz × R_{10}	1.12^{*}	0.85	0.99	0.65	0.82	37^{*}	4^{ns}
Munia/Kauz × R_{16}	1.05	0.93	0.75	1.03	0.89	18^{*}	4^{ns}
TJB368.251/Buc//V81608 × R_{18}	1.01	0.95	0.94	0.85	0.90	13^{ns}	6^{ns}
Varona/Cno//Kauz × R_{20}	0.96	0.86	0.78	0.93	0.86	12^{ns}	1^{ns}
Mean	1.05	0.87	0.81	0.82			
LSD (5%)	0.194						
CV (%)	13.36						
Paired t $_{(n-1)}$ for CMS and CHA	6.15^{**}						

*,** Significant at the 0.05 and 0.01 levels of probability, respectively; ns: Non-significant

Table 4

Relative performance of CMS/CHA facilitated hybrids and parental checks for days to 50% ear emergence

Hybrids	CMS	CHA	B	R
HD2687 × R_3	96.00	98.00	101.00	95.67
Dacula/Chagual//Cazo ×R_6	95.33	95.00*	102.00	88.00
Chilero × R_9	96.00*	104.33**	99.33	102.00
Turaco × R_7	92.00*	101.67	103.67	96.00
Kauz/Hevo × R_8	96.67*	102.67	101.67	99.00
PBW448 × R_{11}	95.67*	99.33	102.33	100.00
Kauz*2/MNV//Kauz × R_{10}	98.00*	102.67*	106.67	106.33
Munia/Kauz × R_{16}	93.67	95.00*	98.67	92.33
TJB368.251/Buc//V81608 × R_{18}	92.33	90.33*	103.67	97.67
Varona/Cno//Kauz × R_{20}	95.33*	101.00**	92.67	94.33
Mean	95.10	99.00	101.17	97.13
LSD (5%)	3.426			
CV (%)	2.15			

Paired $t_{(n-1)}$ for CMS and CHA: –3.32** for CHA and B, –1.13^{ns} for CMS and B, for CMS and R, for CHA and R; *,** Significant at the 0.05 and 0.01 levels of probability, respectively

Plant height

All the CMS hybrids were taller than their B parents. This was in agreement with the results of Virmani and Edwards (1983) and Li et al. (1997). The mean plant height in CMS hybrids ranged from 91 cm to 98.67 cm, whereas it ranged from 80.33 cm to 94.67 cm in CHA hybrids (Table 5). The overall mean height of the CMS hybrids was higher than that of the CHA hybrids and the B parents. There was no hybrid shorter than the shortest R parent (69 cm) or taller than the tallest R parent (109.33 cm). The CMS hybrids were consistently taller than 90 cm; on the other hand the heights of the CHA hybrids were between 80 cm and 90 cm except for Chilero × R_9. However, the parents showed variable heights ranging from 60 to over 100 cm. The grand mean of the CHA hybrids (86.90 cm) approached that of the grand mean of the B parents (83.534 cm). Similarly, using a photoperiod-sensitive cytoplasmic male sterile form of *Ae. crassa*, Murai (1998) reported significant differences between wheat hybrids for plant height.

Yield components

Weight of ten ears

No significant differences were observed between the CMS- and CHA-based hybrids with respect to this character. The overall average performance in weight of ten ears was equivalent in both CMS and CHA hybrids. The paired $t_{(n-1)}$ for the CMS and CHA means was –0.01, which was not significant.

Table 5

Relative performance of CMS/ CHA facilitated hybrids and parental checks for plant height (cm)

Hybrids	CMS	CHA	B	R
HD2687 × R_3	92.33^*	83.67	78.67	84.33
Dacula/Chagual//Cazo × R_6	93.33	89.33	87.33	69.33
Chilero × R_9	98.67	94.67	90.67	99.00
Turaco × R_7	96.67^*	80.33	81.00	93.67
Kauz/Hevo × R_8	98.33^*	89.00	81.00	94.67
PBW448 × R_{11}	91.33^*	84.00	80.67	91.67
Kauz*2/MNV//Kauz × R_{10}	94.67^*	84.67	86.00	97.33
Munia/Kauz × R_{16}	93.00	88.67	85.67	90.33
TJB368.251/Buc//V81608 × R_{18}	92.67^*	86.67	87.00	109.33
Varona/Cno//Kauz × R_{20}	91.00	88.00	77.33	91.00
Mean	94.20	86.90	83.53	92.06

LSD (5%): 5.846

CV (%): 4.03

Paired $t_{(n-1)}$ for CMS and CHA, 5.74^{**}; for CHA and B, 2.78^*; for CHA and R, -1.47^{ns}.

$^{*, **}$ Significant at the 0.05 and 0.01 levels of probability, respectively

Thousand-grain weight

Four out of the 10 CMS-based hybrids were significantly superior to their corresponding CHA hybrids (Table 6). The grand mean thousand-grain weight was greater in CMS hybrids (38.39 g) than that of the CHA-based hybrids (35.85 g). Using a photoperiod-sensitive cytoplasmic male sterile form of *Aegilops crassa* Murai (1998) reported significant differences for thousand-grain weight. Liu et al. (1995) found that grain weight was generally lower in A lines than B lines as the *T. timopheevii* cytoplasm had an unfavourable effect on grain filling. In the present experiment, however, the A line hybrids (involving *T. timopheevii* cytoplasm) were found to have higher thousand-grain weight than the corresponding B line (CHA-treated female parent) hybrids.

Number of grains per spike

Differences were observed between the CMS and CHA hybrids for number of grains per spike (Table 7). The highest number of grains per spike was observed in CHA hybrids and the lowest in CMS-based hybrids. The lower number of grains per spike in CMS hybrids may be attributed to the incomplete fertility restoration. Similarly, in a comparison of hybrids with *T. timopheevii* and *T. aestivum* (hand-emasculated) cytoplasm, Walcott (1985) observed significant differences in grain yield, which he concluded were due to the significantly reduced seed set in the *T. timopheevii* hybrid. The highest number of grains per spike observed in CHA hybrids was in conformation with the results of Borghi et al. (1988).

Table 6

Relative performance of CMS/CHA facilitated hybrids and parental checks for thousand-grain weight (g)

Hybrids	CMS	CHA	B	R
HD2687 × R_3	38.97*	32.62	30.72	34.78
Dacula/Chagual//Cazo ×R_6	36.62*	30.49	30.93	21.32
Chilero × R_9	38.06	34.34	38.39	31.68
Turaco × R_7	40.27*	34.35	34.26	34.36
Kauz/Hevo × R_8	38.60	38.73	28.72	38.14
PBW448 × R_{11}	37.49	37.01	30.23	32.82
Kauz*2/MNV//Kauz × R_{10}	37.53*	32.58	30.54	31.68
Munia/Kauz × R_{16}	39.50	42.18	27.98	35.36
TJB368.251/Buc//V81608 × R_{18}	39.03	40.07	31.62	33.32
Varona/Cno//Kauz × R_{20}	37.76	36.14	33.11	28.62
Mean	38.38	35.85	31.65	32.21
LSD (5%)	4.603			
CV (%)	8.20			

Paired t $_{(n-1)}$ for CMS and CHA: 2.42; * Significant at the 0.05 level of probability

Table 7

Relative performance of CMS/CHA facilitated hybrids and parental checks for number of grains per spike

Hybrids	CMS	CHA	B	R
HD2687 × R_3	51.90	51.40	43.96	46.06
Dacula/Chagual//Cazo ×R_6	45.90	44.73	39.06	34.66
Chilero × R_9	54.66	47.83	41.16	54.30
Turaco × R_7	53.90	54.20	45.60	58.40
Kauz/Hevo × R_8	49.30*	59.50	58.46	49.90
PBW448 × R_{11}	46.70	54.63	54.86	59.80
Kauz*2/MNV//Kauz × R_{10}	48.36*	64.50	56.16	46.00
Munia/Kauz × R_{16}	55.16	63.33	60.80	60.00
TJB368.251/Buc//V81608 × R_{18}	46.40	52.00	58.93	39.46
Varona/Cno//Kauz × R_{20}	44.50*	69.10	56.76	47.10
Mean	49.68	56.12	51.58	49.57
LSD (5%)	8.244			
CV (%)	9.79			

Paired t $_{(n-1)}$ for CMS and CHA: – 2.21; * Significant at the 0.05 level of probability

Tillers per metre

No significant differences were observed between CMS- and CHA-based hybrids with respect to this character. The highest number of tillers per metre was observed in CMS hybrids and the lowest in the B parents. In general, CMS-based hybrids showed better tillering (143.93) than CHA-based hybrids (125.63) and their parents (120.58 and 131.00 for the B and R lines, respectively). The paired t $_{(n-1)}$ for CMS and CHA means was 9.16, which was highly significant.

Discussion

In this experiment the CMS-based hybrids showed superiority in performance to their CHA-based counterparts. They exhibited significantly higher grain yield because of higher seed weight and profuse tillering. These traits, previously reported 24 and 19 times, respectively, as the principal sources of heterosis (Pickett, 1993), compensated for the low number of grains per spike and the low weight of ten ears that were caused by incomplete fertility restoration in these hybrids. On the other hand, the CHA-based hybrids had lower grain weight and number of tillers, resulting in lower grain yield performance. This may in turn be attributed to the toxicity of the CHA being carried over to the hybrids.

Tsunewaki (1980) examined the agronomic characters of 15 F_1 hybrids produced using the *T. timopheevii* system and found that, on average, all 15 hybrids showed delayed heading, taller plant height and higher thousand-grain weight in comparison with the check cultivar, Norin 61. This was in conformation with the results for CMS-based hybrids in the present experiment, except that most of them were earlier than their earlier parents.

The comparison of the two systems of male sterility in wheat, using a universal CMS (*T. timopheevii*) and a non-commercially used CHA (CH9832), will be of assistance to wheat breeders in deciding which path to follow. At the start of the experiment it was hard to find a CHA better than CH9832, which does not fulfil most of the criteria of an ideal CHA, so the results of this specific experiment were in favour of the CMS system. However, the use of ideal CHAs such as GENESIS® may reverse the situation. The use of CHAs has many advantages over the CMS system, but these are only manifested when ideal CHAs are available.

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References

Adugna, A., Nanda, G. S., Singh, K., Bains, N. S. (2004): A comparison of cytoplasmic and chemically-induced male sterility systems for hybrid seed production in wheat (*Triticum aestivum* L.). *Euphytica*, **135**, 297–304.

Bailey, T. B., Qualset, C. O., Cox, D. F. (1980): Predicting heterosis in wheat. *Crop Sci.*, **20**, 339–342.

Barbosa, N. J. F., Sorrells, M. E., Cisar, G. (1996): Prediction of heterosis in wheat using coefficient of parentage and RFLP-based estimates of genetic relationship. *Genome*, **39**, 1142–1149.

Borghi, B., Perenzin, M., Nash, R. J. (1988): Agronomic and quantitative characteristics of ten bread wheat hybrids produced using a chemical hybridizing agent. *Euphytica*, **39**, 185–194.

Briggle, L. W. (1963): Heterosis in wheat – a review. *Crop Sci.*, **3**, 407–412.

Bruns, R., Peterson, C. J. (1998): Yield and stability factors associated with hybrid wheat. *Euphytica*, **100**, 1–5.

Carver, B. F., Smith, E. L., England, H. O. (1987): Regression and cluster analysis of environmental responses of hybrid and pure line winter wheat cultivars. *Crop Sci.*, **27**, 659–664.

Cisar, G., Cooper, D. B. (2002): Hybrid wheat. pp. 157–173. In: Curtis, B. C., Rajaram, S., Gomez-Macpherson, H. (eds.), *Bread Wheat: Improvement and Production*. Plant Production and Protection Series No. 30, FAO, Rome.

Cukadar, B., Van Ginkel, M. (2001): Yield potential of bread wheat hybrids produced by Genesis. *Kronstad Symp. Poster List*, CIMMYT.

Johnson, V. A. (1977): The role of wheat in America's future. In: *Agronomists and Food: Contributions and Challenges*. Amer. Soc. Agron., Madison, pp. 37–44.

Li, Y. C., Peng, J. H., Liu, Z. Q. (1997): Heterosis and combining ability for plant height and its components in hybrid wheat with *Triticum timopheevi* cytoplasm. *Euphytica*, **95**, 337–345.

Liu, Z. Q., Li, Y. C., Liu, Z. Q. (1995): Studies on grain weight heterosis of hybrid wheats with *T. timopheevi* cytoplasm. *Acta Agron. Sinica*, **21**, 57–63.

Lucken, K. A., Johnson, K. D. (1989): Hybrid wheat status and outlook. In: IRRI, *Hybrid Rice*. Proc. Int. Symp. Hyb. Rice. 6–10 Oct 1986. Changsha, Hunan, China, pp. 243–255.

Murai, K. (1998): F_1 seed production efficiency by using photoperiod-sensitive cytoplasmic male sterility and performance of F_1 hybrid lines in wheat. *Breed. Sci.*, **48**, 35–40.

Peterson, C. J., Moffat, J. M., Erickson, J. R. (1997): Yield stability of hybrid vs. pure line hard winter wheats in regional performance trials. *Crop Sci.*, **37**, 116–120.

Pickett, A. A. (1993): Hybrid wheat – results and problems. *Plant Breed.*, **15** (Supplement), 1–259.

Pickett, A. A. (1998): Wheat. pp. 257–281. In: Banga, S. S., Banga, S. K. (eds.), *Hybrid Cultivar Development*. Narosa Publishing House, New Delhi, India.

Tandon, J. P. (1995): Present status and future prospects of hybrid wheat. pp. 159–164. In: Rai, M., Mauria, S. (eds.), *Hybrid Research and Development*. The Indian Soc. Seed Tech., New Delhi, India.

Tsunewaki, K. (1980): Basic studies on hybrid wheat breeding utilizing the *timopheevi* cytoplasm and *Rf3* gene. Summary of the results. *Seiken Ziñhó*, **29**, 40–56.

Virmani, S. S., Edwards, I. B. (1983): Current status and future prospects for hybrid rice and wheat. *Adv. Agron.*, **36**, 145–214.

Walcott, J. J. (1985): The effect of the *Triticum timopheevi* nucleo-cytoplasmic system on the performance of an F_1 hybrid wheat. *Aust. J. Agric. Res.*, **36**, 553–557.

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Short communication

EFFECT OF 2,4-D AND INOCULATION WITH *Azorhizobium caulinodans* ON MAIZE

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Research over the last few years has shown that inoculation with nitrogen-fixing bacteria of the genus *Azorhizobium* presents an alternative for (or supplement to) chemical fertilization, mainly due to the capability of the bacteria to produce plant growth-promoting hormones. The *Azorhizobium caulinodans* strain ORS 571 in combination with 2,4-D was able to colonize the root interior of an Indian maize cultivar. After transplanting to pots, it was noticed that nodulated and *Azorhizobium*-treated plants showed higher chlorophyll content in the leaf and enhanced nitrate reductase activity, leading to higher yield as compared to the control plants (non-nodulated). A plant growth-promoting effect was clearly visible in all inoculated plants examined. Nodulated plants treated with *Azorhizobium* had higher physiological activities as compared to plants treated only with *Azorhizobium*. *Azorhizobium* therefore creates potentially better symbiosis in the form of *para*-nodules and promotes a higher level of nitrogen fixation, leading to better growth and plant development, with reduced requirements for chemical fertilizers.

Key words: *Azorhizobium caulinodans*, 2,4-D, nodulation, nitrogen fixation, grain yield, maize

Introduction

Although various attempts have been made to transfer biological nitrogen fixation (BNF) from legumes to non-legumes, there are other ways of achieving BNF that are perhaps more promising. Over the past two and half decades, farmers have become increasingly dependent on chemical sources of N for obtaining higher yields to meet the demands of the burgeoning population. Keeping in view the increasing N requirement and the low utilization efficiency, as well as the severe negative environmental impacts of N fertilizers, it is time to think of alternative sources of N. If a BNF system were developed in maize plants, it would enhance the N supply potential, as the fixed N would be

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available directly to the plants with little or no loss. It would also reduce both the cost of applying nitrogenous fertilizers and their detrimental effect on the environment. The use of microorganisms to influence the yield components of field-grown crops has always been neglected in intensive farming systems. Biofertilizers like *Azorhizobium* can act as perpetually renewable inputs, helping to achieve better crop nutrient management and to maintain soil health. A well-known example of this is the use of the *Azotobacter chroococcum* preparation 'Azotobacterin', which has been propagated in the U.S.S.R. since 1926 (Mishustin, 1970). *Azorhizobium caulinodans* is particularly interesting because it can grow and fix atmospheric nitrogen without the addition of nitrogen under free-living conditions (Dreytus et al., 1983). Among the plant hormones, 2,4-D, a synthetic auxin, was found to be the best for inducing nodular outgrowth in cereals (Ridge et al., 1993; Christiansen-Weniger and Vanderleyden, 1994). Wilde (1951) also observed that the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) induced nodule-like outgrowths on the roots of beans. The present study was undertaken as an attempt in this direction using the synthetic auxin 2,4-D and the N_2-fixing bacteria *Azorhizobium caulinodans* (ORS 571). The symbiotic relationship between plants and bacteria was analysed in terms of the growth and yield patterns of transplanted maize in pots after nodule induction under laboratory conditions.

Materials and methods

Bacterial culture, plant host and induction of p-nodules

Seeds of *Zea mays* L. cv. Kiran were surface sterilized using 0.5% $HgCl_2$ for 2 minutes and rinsed thoroughly with distilled water. The seeds were germinated on sterile, acid-washed gravel culture at 25°C for 5–6 days. Uncontaminated seedlings were grown *in vitro* in glass tubes (length 20 cm, diameter 3 cm, one seedling per tube) containing sterile N-free Hoagland solution at 25°C under continuous lighting (600 $\mu E\ m^{-2}s^{-1}$) . The glass tubes were covered with black paper to avoid the exposure of the root zone to light. Bacterial inoculation was performed two days after transferring the seedlings to glass tubes with 0.1 ml of *Azorhizobium caulinodans* (ORS 571) culture, grown at room temperature for 24 hours and containing 10^7 to 10^8 cells/ml, and a sufficient quantity of a sterile 2,4-D solution to give a final concentration of 1.0 ppm (2,4-D). The following four treatments were applied in nitrogen-free Hoagland solution:

T_1 – Control
T_2 – 2,4-D
T_3 – *Azorhizobium caulinodans*
T_4 – 2,4-D + *Azorhizobium caulinodans*

Growth and yield parameters

After nodulation under laboratory conditions, the two-week-old seedlings were transferred to pots (50×50×50 cm) in a net house under natural conditions. Each pot was filled with 45 kg of soil and well decomposed FYM was added at the rate of 2 kg per pot. Three healthy seedlings were maintained in each pot in a replicated trial and the growth parameters were measured at 30 and 60 days after transplanting (DAT). The chlorophyll content in the leaves was estimated using DMSO (dimethyl sulphoxide) (Hiscox and Israelstam, 1979). The tubes containing leaf tissues and DMSO were kept in a hot air oven adjusted to 65°C for about 3 hours. The absorbance of the chlorophyll extract was measured at 663 and 645 nm using a Spectronic 20 instrument (Labequip, Canada). The nitrate reductase activity was measured in the leaves using the method of Klepper et al. (1971). The first fully expanded leaf from the top was used for assaying the nitrate reductase activity. Leaf material (0.3 g) was put into ice cold infiltration tubes containing 2.5 ml KNO_3 and 2.5 ml phosphate buffer (pH 7.5). The tubes were then passed through a vacuum for 5 minutes so that the nitrate infiltrated the leaf tissue, and then incubated at 33°C for 1 h in the dark. For the assay of enzyme activity, 0.2 ml of reaction mixture from each tube was transferred to another set of test tubes containing 1.0 ml of sulphanylamide and 1.0 ml of NEDD (naphthylethylene diamine dihydro-chloride). The absorbance of the reaction mixture was measured at 540 nm using a Spectronic 20 instrument. The yield components were measured after harvest and the nitrogen content of the grain and stover at maturity was estimated using an auto-analyser (Technicon, USA).

Statistical analysis

The statistical analysis was done following the standard procedure described by Panse and Shukhatme (1967). All observations are means of three replicates and comparisons of treatment means were made at the 5% confidence level.

Results and discussion

When treated with 2,4-D (1.0 ppm) maize seedlings developed nodule-like knots along the primary roots and club-shaped tumours at their root tips, compared with untreated plants. However, well-developed nodule-like tumour knots that can best be described as modified lateral roots (*para*-nodules) emerged only when the plants were inoculated with *Azorhizobium* along with 2,4-D. The total chlorophyll concentration was maximum in seedlings treated with both *Azorhizobium* and 2,4-D at both 30 and 60 DAT (Table 1). Maize seedlings treated with *Azorhizobium* alone also showed a higher chlorophyll concentration compared with the control and seedlings treated with 2,4-D. The nitrate reductase activity was also higher in plants treated with *Azorhizobium* either alone or in combination with 2,4-D than in the uninoculated control at both 30 and 60 DAT (Table 1). These results are in accordance with the earlier findings of Van Nieuwenhove et al. (2001) in rice plants, in which *Azorhozobium* enhanced the nitrate reductase activity.

Table 1

Effect of 2,4-D and inoculation with *Azorhizobium caulinodans* on leaf chlorophyll content (mg/g F.W.) and nitrate reductase activity (μ mol NO_2/g F.W./h) in nodulated maize after transplantation to pots

Treatments	Chlorophyll content		Nitrate reductase activity	
	30 DAT	60 DAT	30 DAT	60 DAT
Control	1.35	2.46	0.03	0.47
2,4-D	1.12	2.18	0.03	0.38
Azorhizobium	1.28	2.70	0.05	0.86
2,4-D+*Azorhizobium*	1.35	2.97	0.07	0.98
CD at 5%	0.12	0.04	0.01	0.33

DAT – Days after transplanting

Data pertaining to the yield parameters (Table 2) showed that inoculation with *Azorhizobium caulinodans* alone or along with 2,4-D contributed to an increase in yield. The combination of 2,4-D and inoculation with *Azorhizobium* produced a higher number of ears per plant, with increased ear length, ear weight and a higher number of grains per ear. The yield increases obtained in inoculated plants have been attributed to biological N_2 fixation and may also be due to the production of growth substances by the colonizing bacteria. The increased N supply through N_2 fixation, higher nutrient uptake (Subba Rao et al., 1985; Panwar, 1993) and higher chlorophyll content in the inoculated plants (Panwar and Elanchezhian, 1998) might have contributed to the increase in grain yield. The nitrogen content in the grain and stover was also increased by inoculation with *Azorhizobium*, the greatest N content being observed in plants treated with both 2,4-D and *Azorhizobium* (Table 3). The enhanced N content in grain and stover due to inoculation confirms N_2 fixation and the transfer of fixed N. The increased N content may be attributed to greater mineral uptake, nitrogenase activity and nitrate reductase activity (Kennedy and Tchan, 1992; Panwar, 1993).

Table 2

Effect of 2,4-D and inoculation with *Azorhizobium caulinodans* on yield and yield components in nodulated maize after transplantation to pots

Treatments	Ear length (cm)	No. of grains per ear	100-grain weight (g)
Control	12.90	243.33	22.37
2,4-D	9.23	237.67	11.88
Azorhizobium	14.07	293.00	23.12
2,4-D+*Azorhizobium*	16.07	324.33	24.68
CD at 5%	2.23	4.56	1.74

Table 3

Effect of 2,4-D and inoculation with *Azorhizobium caulinodans* on nitrogen content in grain and stover in nodulated maize after transplantation to pots

Treatments	Grain nitrogen content (%)	Stover nitrogen content (%)
Control	0.42	0.25
2,4-D	0.41	0.23
Azorhizobium	0.45	0.27
2,4-D+*Azorhizobium*	0.47	0.30
CD at 5%	0.01	0.08

The overall decrease in all the physiological parameters in seedlings treated only with 2,4-D was due to poor root and shoot growth, root morphogenesis and nodulation without nitrogenase activity and leghemoglobin (data not shown) and resulted in reduced biomass production, grain yield and N uptake. Inoculation with N_2-fixing bacteria without any 2,4-D treatment showed no effect on root and shoot growth and nodulation but caused the induction of nitrogenase activity and leghemoglobin at a lower rate, which enhanced the yield compared to the uninoculated treatments. However, inoculation with N_2-fixer in addition to 2,4-D treatment caused nodulation and the induction of nitrogenase activity and leghemoglobin content, leading to an increase in N_2 fixation. This contributed to higher chlorophyll content and NR activity, leading to higher grain yield and increased N content in the grain and stover.

Based on the observations in the present study it can be concluded that *Azorhizobium* inoculation benefits plant growth and also increases yield by improving root development and uptake. The improvement in plant growth after the application of biofertilizers can be attributed to an increase in the number of lateral roots and root hair formation (Jain and Patriquin, 1985), water and mineral uptake (Okon and Kapulnik, 1986). Biological nitrogen fixation by *Azorhizobium* also contributes a significant amount of nitrogen to the plants, thereby resulting in a saving of Na fertilizers.

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References

Christiansen-Weniger, C., Vanderleyden, J. (1994): Ammonium-excreting *Azospirillum* sp. become intracellularly established in maize (*Zea mays*) para-nodules. *Biol. Fertil. Soils*, **17**, 1–8.

Dreytus, B., Elmerich, C., Dommergues, Y. R. (1983): Free-living *Rhizobium* strains able to grow on N_2 as the soil nitrogen source. *Appl. Environ. Microbiol.*, **45**, 711–713.

Hiscox, J. D., Israelstam, G. F. (1979): A method for extraction of chlorophyll from leaf tissues without maceration. *Can. J. Bot.*, **57**, 1332–1334.

Jain, D. K., Patriquin, D. G. (1985). Characterization of a substance produced by *Azospirillum*, which causes branching of wheat root hairs. *Can. J. Microbiol.*, **31**, 206–210.

Kennedy, I. R., Tchan, Y. T. (1992): Biological nitrogen fixation in non-leguminous field crops: Recent advances. *Plant and Soil*, **141**, 93–118.

Klepper, L. A., Flesher, D., Hageman, R. H. (1971): Generation of reduced nicotinamide adenine di-nucleotide for nitrate reduction in green leaves. *Plant Physiol.*, **48,** 580–590.

Mishustin, E. N. (1970): The importance of non-symbiotic nitrogen-fixing micro-organisms in agriculture. *Plant and Soil*, **32**, 545–554.

Okon, Y., Kapulnik, Y. (1986): Development and function of *Azospirillum*-inoculated roots. *Plant and Soil*, **90**, 3–16.

Panse, V. G., Shukhatme P. V. (1967): *Statistical Procedures for Agricultural Workers*. ICAR Publication, New Delhi.

Panwar, J. D. S. (1993): Response of VAM and *Azospirillum* inoculation to water status and grain yield in wheat under stress conditions. *Indian J. Plant Physiol.*, **36**, 41–43.

Panwar, J. D. S., Elanchezhian, R. (1998): Effect of 2,4-D and *Azospirillum brasilense* on growth and yield in the nodule induced transplanted wheat. *Indian J. Plant Physiol.*, **3**, 143–146.

Ridge, R. W., Ride, K. M., Rolfe, B. G. (1993): Nodule like structures induced on the roots of rice seedlings by the addition of synthetic auxin 2,4-dichlorophenoxy acetic acid. *Aust. J. Plant Physiol.*, **20**, 705–717.

Subba Rao, N. S., Tilak, K. V. B. R., Singh, C. S. (1985): Yield responses of crop to inoculation with *Azospirillum brasilense* in India. *Zbl. Mikrobiol.*, **140**, 97–102.

Van Nieuwenhove, C., Merckx, R., Van Holm, L., Vlassak, K. (2001): Dinitrogen fixation activity of *Azorhizobium caulinodans* in the rice (*Oryza sativa* L.) rhizosphere assessed by nitrogen balance and nitrogen-15 dilution methods. *Biol. Fertil. Soils,* **33**, 25–32.

Wilde, M. H. (1951): Anatomical modifications of bean roots following treatment with 2,4-D. *Am. J. Bot.,* **38**, 79–91.

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Book reviews

J. SUTKA: Plant Cytogenetics. 2004. Mezőgazda Kiadó. Budapest, Hungary. 232 pages, ISBN 963-286-170-1

Twenty-five years ago, in 1980, the book entitled "Cytogenetics", written by József Sutka, was published in Budapest by the agricultural publishers Mezőgazdasági Könyvkiadó. At the time this was the only book on this topic in Hungarian, so it was in great demand for a long period both among university students and postgraduates and among specialists working on the breeding and genetics of agricultural crops. However, the enormous advances made in the fields of genetics and genetic methodology in recent decades have made it necessary to revise the book to include new information. In this new volume, Plant Cytogenetics, the author has retained all the basic material required for a discussion on cytogenetics, but has added a great deal of new material, including several new chapters. These new chapters contain information on molecular genetics and genomics, which are closely connected with cytogenetics, as these techniques often use basic materials developed using conventional cytogenetic methods.

The first chapter deals with the behaviour of chromosomes during the mitotic cell cycle. In addition to the usual description of mitosis, the latest results on the regulation of the cell cycle are also included, together with a discussion of special types of chromosomes. The following chapter describes the behaviour of chromosomes during meiosis, including detailed information on the structure of the synaptonemal complex and on genetic mapping based on recombination frequency. The presentation of the structure and doubling of eukaryotic chromosomes in chapter three is much more detailed than in the earlier book and emphasis is placed on new discoveries made in recent decades both when describing chromosome structures and when introducing the subject of artificial chromosomes. The fourth and fifth chapters deal with structural and numerical changes in the chromosomes, while the subject of the sixth chapter is gene localisation and gene transfer by means of chromosome manipulation. This includes the description of gene localisation with the aid of the deletion lines developed in recent decades, but the chapter also includes information on the results achieved in the field of alien gene transfer.

Chapter 7 deals with extranuclear inheritance, providing information on the genomic structure of chloroplasts and mitochondria, while Chapter 8 is an introduction to molecular genetic methods and genomics, covering a broad area ranging from the structure of plant genes, through the establishment of gene libraries and DNA sequencing to a discussion of genomics. This same chapter also deals with various molecular marker techniques and with molecular cytogenetic methods. These two chapters were not part of the previous volume, containing as they do results obtained over the last 10–20 years. The book closes with a brief description of various methods of chromosome analysis.

The large number of good quality figures, many of which are cytogenetic photographs taken in the author's own laboratory at the Agricultural Research Institute of the Hungarian Academy of Sciences in Martonvásár, are of great assistance in following and understanding the arguments, while also confirming the author's profound knowledge of his subject, making him an internationally renowned expert.

The book was published with financial assistance from the Ministry of Education and is intended mainly as a textbook for students at agricultural universities and colleges. Nevertheless, it can be warmly recommended to all those working in the field of plant biology who would like to gain a broader view of plant cytogenetics and related molecular genetic research. The book will be a useful manual for plant breeders and geneticists.

The publication of József Sutka's new book will also be of importance as an authentic source of accepted Hungarian terms for the many novel concepts arising constantly in the rapidly developing field of genetics.

M. MOLNÁR-LÁNG

Z. TUBA (Editor): Ecological Responses and Adaptations of Crops to Rising Atmospheric Carbon Dioxide has been co-published simultaneously as Journal of Crop Improvement, Volume 13, Number 1/2 (#25/26) 2005. The Haworth Press, Inc., Binghamton, NY, USA 414 pages; ISBN-13:978l-1-56022-121-0

The atmospheric CO_2 concentration has been on the increase ever since the industrial revolution, and this exerts both direct and indirect effects on plants, animals and human beings.

The book entitled "Ecological responses and adaptations of crops to rising atmospheric carbon dioxide" is an excellent resumé of current knowledge on the predicted effects of a rise in the atmospheric CO_2 concentration on agricultural and horticultural plants, trees and pastures. It also deals with the relationship between high atmospheric CO_2 concentration and other factors involved in climate change, such as high temperature, drought, ozone and nitrogen supplies.

It is important to note that the volume contains not only experimental data, but also the results achieved with simulation models that can be used to estimate expected changes in agricultural crops as the result of higher atmospheric CO_2 concentration and climate change.

The high scientific standard of the individual chapters, the descriptions of experimental and simulation methods, the illustration of the results in tables and figures, the summaries and the references will be of use not only to scientists working in this field, but also as a textbook for PhD students and undergraduates and as a manual for ecologists and agriculturalists.

The book not only gives a comprehensive review of present knowledge, but also raises new questions that must be addressed in the future if sustainable agricultural production is to be achieved.

N. HARNOS

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Obituary

GYULA PÁL
(1929–2005)

Gyula Pál, who was born in Kálóz, Hungary in 1929 and worked in the Agricultural Research Institute of the Hungarian Academy of Sciences for 32 years, died on 4 July 2005 in his 77th year.

He began his university studies at the University of Agricultural Sciences in Gödöllő, but had to abandon his course for political reasons. He later continued his studies at Eötvös Loránd University, Budapest, obtaining a first class degree in biology in 1955.

After short periods working in the Botanical Research Institute of the Hungarian Academy of Sciences in Vácrátót and in the Research Institute of the Paper Industry, the political situation in Hungary again caused a disruption of his career, but in 1957 he finally obtained a post as a research associate in the Plant Genetics Department of the Martonvásár institute, where he remained until his retirement.

Gyula Pál had a wide range of interests. In addition to his scientific work on plant anatomy, he also acted as editor of the journal Acta Agronomica Hungarica, while his hobby was the collection of rare books.

His research included investigations on the phenomenon of heterosis, pollen formation in egg-plants, and the development of the spike in wheat and wheat–*Agropyron* hybrids. The results obtained in these fields were published mainly in the Hungarian journals Biológiai Közlemények, Növénytermelés and Acta Agronomica Hungarica. His principal collaborators were Erna Rajki, János Pletser and Dezső Szalay.

Together with the Editor-in-Chief, Sándor Rajki, he played an important role in increasing the circulation of Acta Agronomica Hungarica, which was subscribed to by over 300 research institutes and universities in the 1960s and 1970s. After the late 70s he was only involved in editing the Forum column of the journal, while in the 1980s, until his retirement in 1989, most of his time was spent in preparing thematic reports on the research carried out in the institute for the Academy of Sciences.

Gyula Pál did not speak any foreign languages, but he had an intimate knowledge of the finer points of the Hungarian language, as demonstrated by the style of his research papers. He devoted considerable time and energy to teaching the language to the many foreign post-graduates who worked in the Martonvásár institute.

He spent most of his free time reading the works on history and literature in his extremely valuable collection, and was consequently well-versed in these subjects. It was largely due to his efforts, and to his extensive acquaintance among antiquarian booksellers, that some of the books originally forming the library of the Brunszvik family, the builders of the Martonvásár mansion now forming the headquarters of the research institute, were traced and purchased for the Beethoven–Brunszvik Museum maintained by the institute.

Gyula Pál will thus be remembered as a man who made a lasting contribution as a scientist, as the editor of Acta Agronomica Hungarica and as a collector of rare books. He will be sadly missed.

E. PÁLDI

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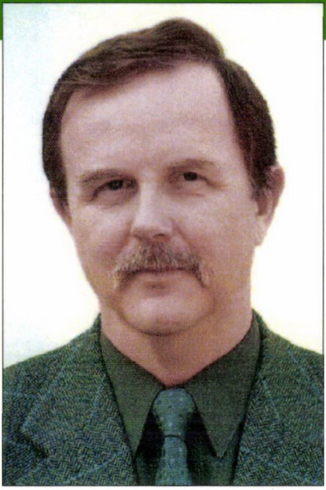

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Acta Agronomica Hungarica, 54(2), p.131 (2006)
DOI: 10.1556/AAgr.54.2006.2.1

PREFACE

Paprika, the major flavour determinant of the Hungarian cuisine, is one of the country's most important export products. Its exceedingly high vitamin C content, the characteristic red colour of spice paprika and its excellent intrinsic value have made Hungarian paprika world famous. The application of modern molecular biology methods will strengthen the ability of Hungarian paprika and sweet pepper breeders to face the intense international competition from South East Asian and Central American breeders, not to mention the current market leaders in Spain and The Netherlands. Advances in biotechnology in Hungary in recent decades have resulted in pioneering achievements in haploid production, paprika tissue culture, and molecular marker-assisted breeding. The practical use of anther culture for paprika has greatly assisted the breeding process. One field of primary interest is the production of homozygous doubled haploid lines due to a chromosome doubling process that may occur spontaneously, but which can also be induced by chemicals. As genotype dependence is a major characteristic of the androgenic response, major breeding lines should be analysed from this aspect. However, one of the most popular cultivars, Fehérözön, gives a significantly better response than other varieties. An optimized protocol for efficient plant regeneration and gene transfer, which includes the use of molecular marker-assisted breeding, is now available for the genetic improvement of paprika. The genetic diversity of Hungarian paprika is the second largest in the world. The exploitation of this biodiversity will help breeders to maintain their market position. The consortium, funded and financed by the Széchenyi program of the national R and D authority, "Gold of Hugaria", has strengthened cooperation between basic and applied research by directly transforming research results into everyday practice. The fact that an industrial partner with great experience in molecular biology was involved in the project is a guarantee of the professional standard of worldwide marketing and of the patenting of the results of R and D work.

This special issue of Acta Agronomica Hungarica contains the major achievements of this research project, together with reviews on the state of the art of pepper breeding, including the history of the pepper plant in the old world.

E. BALÁZS
Consortium head

Acta Agronomica Hungarica, 54(2), pp. 133–140 (2006)
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ANTIOXIDANT CONTENT OF BIO AND CONVENTIONAL SPICE RED PEPPER (*Capsicum annuum* L.) AS DETERMINED BY HPLC

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In the present work, bio and conventional forms of spice red pepper were analysed using various high performance liquid chromatographic (HPLC) systems for their carotenoid, tocopherol and vitamin C contents. The carotenoid pigment was fractionated into free xanthophylls, monoesters, carotenes and diesters with newly developed reversed phase HPLC, while α-, β- and γ-isomers of vitamin E were separated by normal phase chromatography. Ion-pair chromatography on a C-18 column provided good separation and quantification of vitamin C. The peppers included new resistant varieties and hybrids that are essential for bio-production. It was found that crossing new disease-resistant varieties such as Kaldom and Kalorez with susceptible ones such as Rubin and SZ-20 produced resistant hybrids that contained higher levels of quality components compared to the parents, particularly when grown and cultivated under organic farming conditions.

Key words: antioxidant, carotenoid, vitamin C, vitamin E, spice red pepper, liquid chromatography

Introduction

Spice red pepper is one of the most important vegetable crops in Hungary (Somos, 1984; Márkus and Kapitány, 2001). The crop is processed and used in various forms such as ground paprika, paste and oleoresin, which are popular on local and foreign markets.

The organic production of certain horticultural crops is increasing from year to year due to the increasing demand for fertilizer- and pesticide-free products. In conventional production fertilizer and pesticide residues threaten the health of the consumers since they increase the risk of serious diseases such as cancer. Due to the increasing level of environmental pollution and food contamination there is a great need to raise the level of endogenous antioxidants such as carotenoids, vitamin C and vitamin E in fruit and vegetables to neutralize the oxidizing effect of many dangerous pollutants. Red pepper contains considerable amounts of the most effective antioxidant vitamins and

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vital carotenoids that make the crop of special importance from the technological and nutritional points of view (Daood et al., 1996; Márkus et al., 1999).

The purpose of this work was to evaluate the carotenoids and antioxidant vitamins in different varieties and hybrids cultivated under organic and conventional farming conditions.

Materials and methods

Materials

Freshly harvested spice red pepper pods of Rubin, Kaldom, SZ-20, Kalorez and the new hybrids 8 and 3 were obtained from the experimental farms of the Spice Paprika Research Development Co. (Kalocsa, Hungary). The fruits were deseeded and stored at –20°C in vacuumed plastic bags when not immediately analysed.

All chemicals used were analytical grade and purchased from Reanal (Budapest, Hungary). Organic solvents were purchased from Merck (Darmstadt, Germany).

Standard materials such as β-carotene, tocopherols and ascorbic acid were purchased from Sigma (St. Louis, USA).

Sudan-I (Sigma, St. Louis, USA) was used as the internal standard for the quantification of carotenoids. The quantification of tocopherols and ascorbic acid was based on external standards and calibration curves.

Methods

Carotenoid-type pigments and tocopherols were extracted from fresh paprika by a previously described procedure with a slight modification, involving the use of 1,2-dichloroethane instead of carbon tetrachloride (Biacs and Daood, 1994). A 2.5 g sample was crushed in a crucible mortar with quartz sand and 20 ml methanol was added. After standing for 2 min in the dark, the mixture was transferred to a conical flask and the mortar was rinsed with 60 ml of 10:50 methanol-dichloroethane to transfer the remaining pigments to the conical flask. Then a few drops of double distilled water were added to ensure the separation of two layers, followed by shaking for 15 min. The organic solvent phase was filtered through an MN-640 filter paper and the solvent was evaporated under a vacuum. The residues were re-dissolved in 10 ml of the HPLC eluent (C). Before injection on the HPLC column the pigment extract was passed through a 0.45 mm Millipore PTFE syringe filter.

To analyse tocopherols, the pigment extract was saponified with methanolic KOH for 35 min at the boiling point of methanol in the presence of 1 g ascorbic acid. After cooling and the addition of salted water the tocopherol fraction was extracted twice by gentle shaking with 40 ml *n*-hexane. The hexane layers were separated and pooled, then washed three times with distilled water and dried over Na_2SO_4. After vacuum removal of the solvent, the dry residues were collected in 10 ml of HPLC-grade *n*-hexane.

Ascorbic acid was extracted from 2 g of pepper after crushing in a crucible mortar and quantitative transfer to a conical flask with 50 ml of 3% meta-phosphoric acid solution. The mixture was then shaken and filtered through MN-640 filter paper and further cleaned by passing through a 0.45 μm Millipore filter before injection onto the HPLC column.

HPLC conditions

A Nucleosil 100, C-18, 3 μm, 240 mm × 4.6 mm i.d. column was used with gradient elution starting with 100% A (10% water in methanol) changing to 35% A and 65% B (15:35:50 methanol-acetonitrile-isopropanol) in 25 minutes then to 10% A and 90 % B after five minutes, which continued for a further 5 minutes and returned to 100% A in 5 minutes. The flow rate was 1 ml per min. The pigments were detected at 478 nm with a photodiode array detector (Daood and Biacs, 2005).

The tocopherol fraction was separated into different analogues on normal phase Nucleosil 100, 5 μm, 240 mm × 4.6 mm i.d. or Alltima Silica 5 μm, 240 mm × 4.6 mm i.d. columns eluted at a flow rate of 1.2 ml/min with 99.6:0.4 *n*-hexane-absolute alcohol (Speek et al., 1985). The compounds were detected by fluorescence detection at Ex: 295 nm and Em: 320 nm. The detector signals were electronically recorded and integrated.

The separation of ascorbic acid was performed under ion-paired chromatographic conditions using a Nucleodur C18, 5 μm, 240 mm × 4.6 mm i.d. column and a mobile phase consisting of 0.01 M KH_2PO_4:methanol:tetrabutyl ammonium hydroxide (97:3:0.1) at pH 2.8 and a flow rate of 1 ml/min. The detection was performed at 245 nm (Daood et al., 1994).

HPLC instruments

A Waters model Alliance liquid chromatograph, consisting of a Waters 2695 Separation Module, a Waters 2696 photodiode array detector and a Waters 2475 multi-wavelength fluorescence detector, was used. The chromatograph was operated using Empower software. In the case of tocopherol determination, the fluorescence detection was carried out at Ex: 295 nm and Em: 320 nm.

Results and discussion

Determination of quality components

The HPLC profile of carotenoids from an unsaponified extract of biologically ripe red pepper pods is shown in Figure 1. The chromatographic system provided excellent separation of the individual compounds of paprika pigment. The first group of carotenoids, eluted between 0 and 20 minutes, consisted of free xanthophylls such as capsorubin, violaxanthin, capsanthin, cucurbitaxanthin, *cis*-capsanthin, lutein, zeaxanthin, cryptocapsin and β-cryptoxanthin. The second group consisted of monoesters of the afore-mentioned xanthophyll, followed by carotenes not containing oxygen, mainly β-carotene and its *cis*-isomers. The last group contained the chemically most stable carotenoid diesters that are responsible for the colouring capacity and colour stability of spice red pepper pods.

The fat-soluble tocopherols are formed through the same pathway as carotenoid biosynthesis. Therefore, they occur simultaneously with carotenoids in the plant kingdom. Figure 2 shows the HPLC profile of the tocopherols extracted from ripe red pepper peel. The α-vitamer was found to be dominant over other tocopherols such as the β and γ components. In addition to the antioxidant role of α-tocopherol, it has biological activity as vitamin E, which makes it a very important bioactive compound giving functional properties to spice red pepper products and protecting them from oxidation. Red pepper peel also contained small quantities of β- and γ-tocopherols, which are very reactive antioxidants and play an important role in antioxidation processes. Naturally, the level of β-tocopherol is higher than that of γ-tocopherol in seed-free products. When the ratio of β to γ changes in favour of the γ homologue, it indicates that seeds have been ground with the examined samples, since γ-tocopherol is the dominant component found in the seeds.

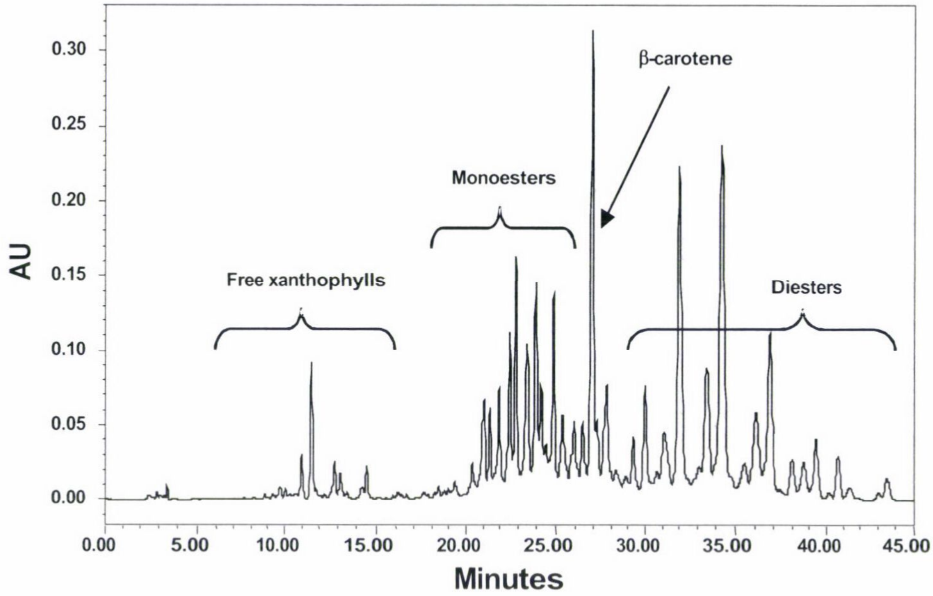

Fig. 1. HPLC separation of carotenoids from red pepper on a Nucleosil 100, 3 μm 250 mm × 4.6 mm i.d. column with gradient elution starting with an aqueous system. For details, see text

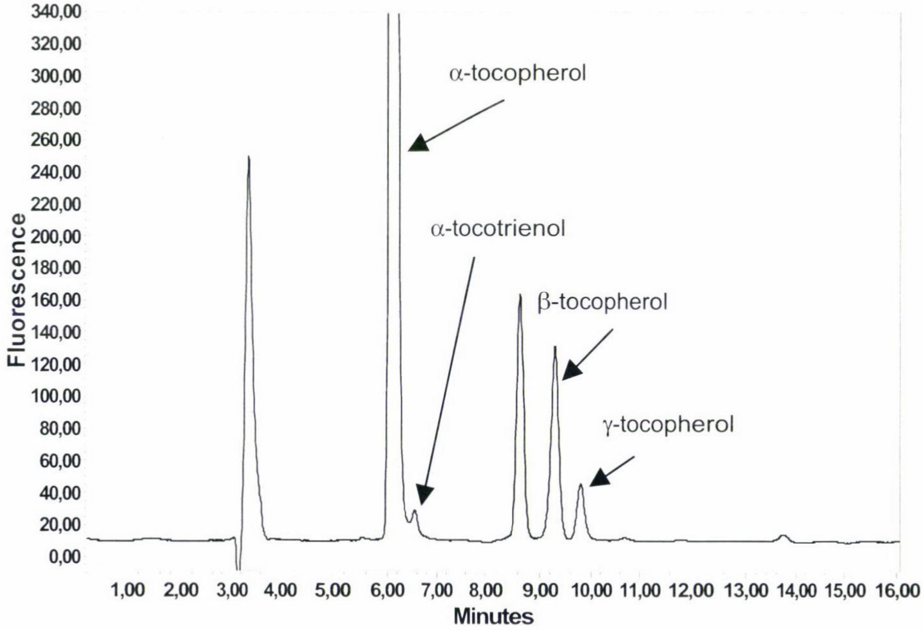

Fig. 2. Normal-phase HPLC separation of tocopherols from red pepper on a Nucleosil 100, 5 μm, 240 mm × 4.6 mm i.d. column. For conditions, see text

The ion-pair HPLC system used for ascorbic acid analysis provided good separation of this antioxidant, which gives the vitamin C activity to red pepper products (Fig. 3). It is well known that dehydroascorbic acid exists together with ascorbic acid in spice red pepper products, but its low absorbance in the UV region of light is below the detection limit. Its accurate determination requires post-column derivatisation to a fluorescent active compound using OPDA (*o*-phenyl-diamine) as described by Bognár and Daood (2000).

Evaluation of resistant varieties and hybrids

The present work focused on changes in the total carotenoids and xanthophyll diesters of red pepper extracts as a function of the hybridisation of resistant and non-resistant varieties and their conventional and bio-production. Figures 4 and 5 show that the hybrid between the non-resistant Rubin and resistant Kaldom (hybrid 8) had total carotenoid and capsanthin diester contents close to that of one of the parents, but when cultivated under bio-production conditions, the pods of this hybrid had higher carotenoid content than the parents, revealing that organic farming conditions resulted in more active carotenogenesis in this hybrid.

The level of fat-soluble antioxidant (Vitamin E) in hybrid 8 was lower than that found in the parents (Rubin and Kaldom). However, cultivation under bio-production conditions improved the α-tocopherol content to the level found in the parents (Fig. 5). When SZ-20 was crossed with Kalorez to produce hybrid 3, both the parents and the hybrid responded positively to the new cultivation conditions and the vitamin E content was significantly increased.

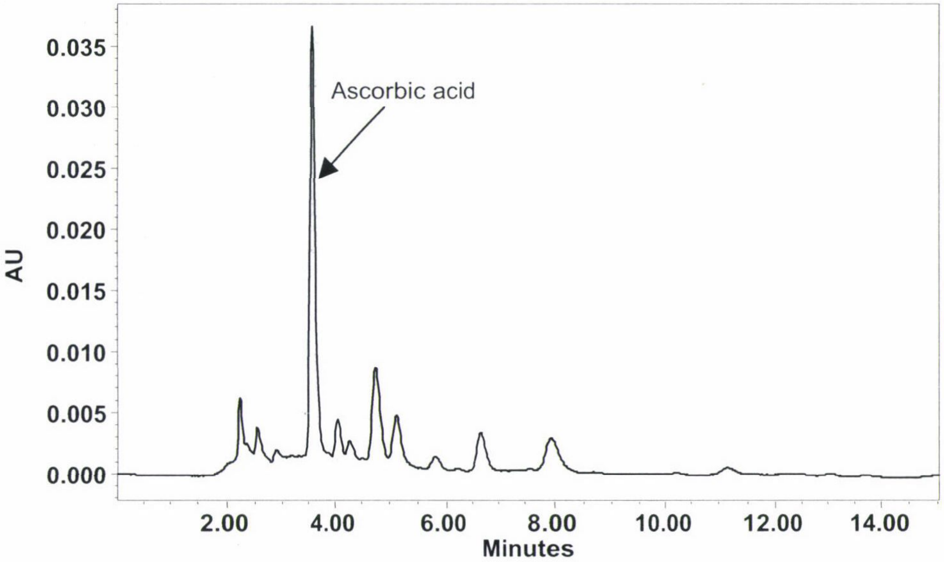

Fig. 3. HPLC chromatogram of vitamin C and other organic acids from red pepper. For conditions, see text

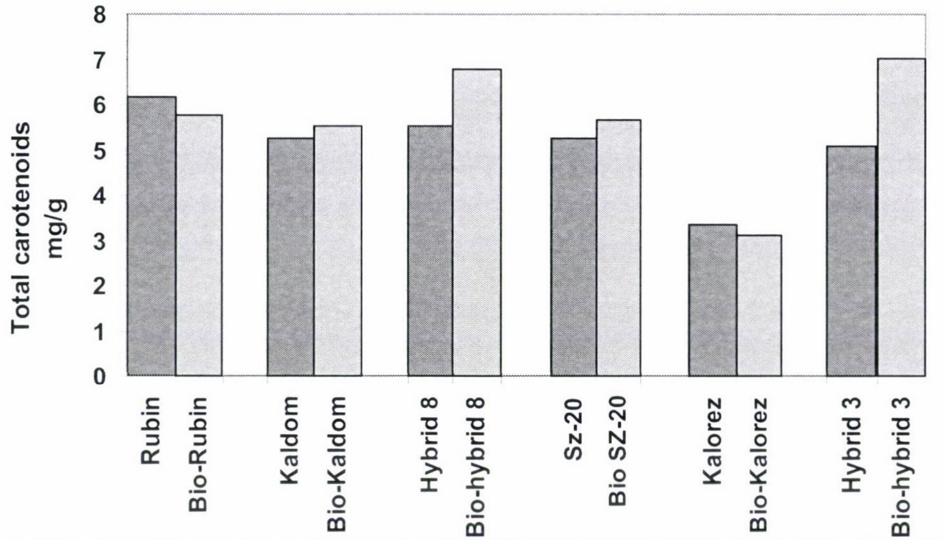

Fig. 4. Total carotenoid content of freshly harvested organic and conventional red pepper of different varieties and hybrids

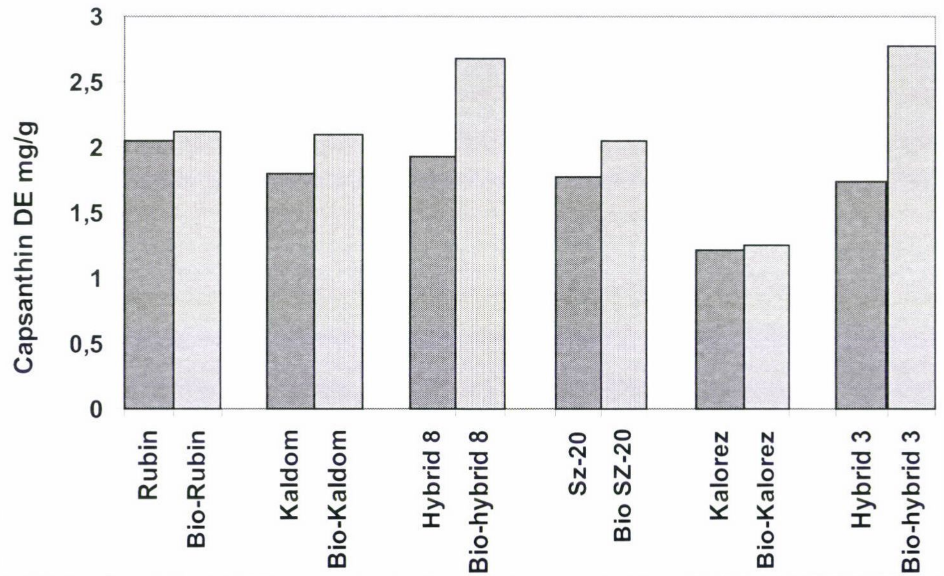

Fig. 5. Capsanthin diester content of freshly harvested organic and conventional red peppers of different varieties

As shown in Figures 6 and 7, crosses between resistant and non-resistant varieties caused a significant increase in the content of vitamins C and E. The great increase in vitamin C content in hybrids 8 and 3 in the case of bio-production is of special interest.

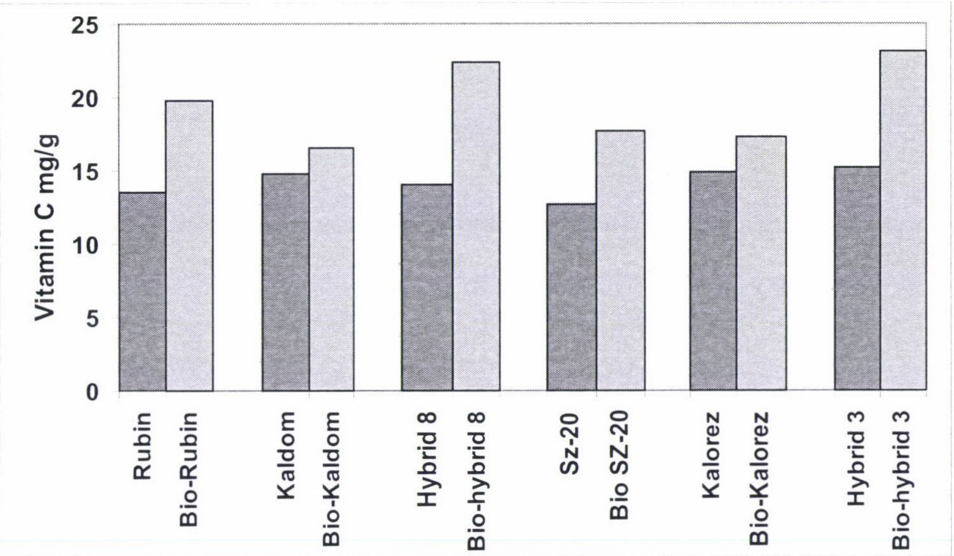

Fig. 6. Vitamin C content of freshly harvested organic and conventional red peppers of different varieties

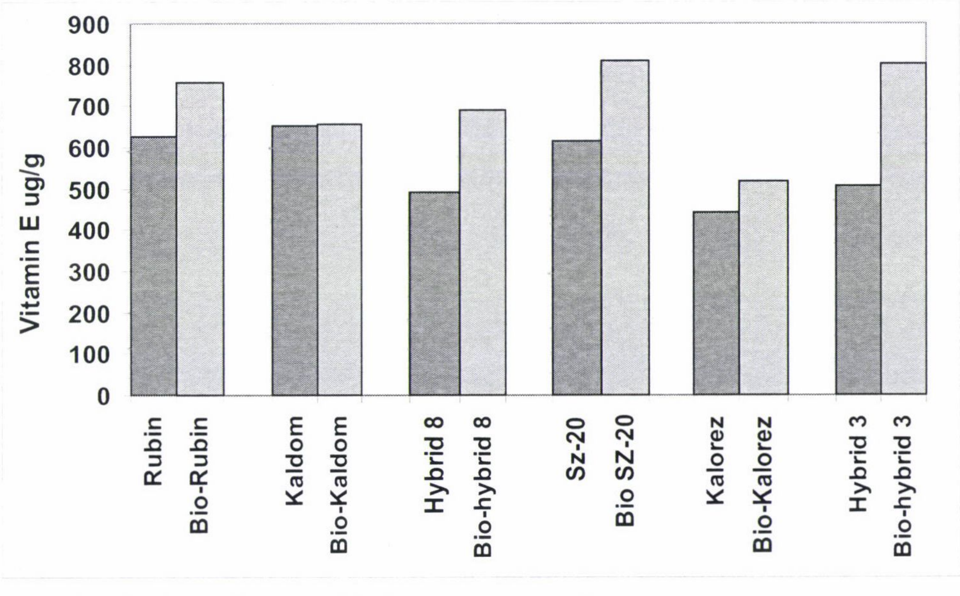

Fig. 7. Vitamin E content of freshly harvested organic and conventional red peppers of different varieties

In conclusion, it can be said that to improve the bacterial resistance and quality attributes of spice red pepper, the hybridisation of the best resistant and non-resistant varieties is necessary. The quality components of paprika can also be improved to a great extent by cultivation under bio-production conditions.

Acknowledgements

The financial support of the Ministry of Education (NKFP 00190/01), the National Scientific Research Fund (OTKA, F038335) and the Hungarian Research and Development Program "Gold of Hungaria", Grant No. 4/006 is greatly appreciated.

References

Biacs, P. A., Daood, H. G. (1994): High-performance liquid chromatography with diode-array detection of carotenoids and carotenoid esters in fruits and vegetables. *J. Plant Physiol.*, **143**, 520–525.

Bognár, A., Daood, H. G. (2000): Simple in-line postcolumn oxidation and derivatization for the simultaneous analysis of ascorbic and dehydroascorbic acids in foods. *J. Chromatogr. Sci.*, **38**, 162–168.

Daood, H. G., Biacs. P. A., Dakar, M. A., Hajdú, F. (1994): Paired-ion chromatography and photodiode-array detection of vitamin C and organic acids. *J. Chromatogr. Sci.*, **37**, 481–487.

Daood, H. G., Vinkler, M., Márkus, F., Hebshi, E. A., Biacs, P. A. (1996): Antioxidant vitamin content of spice red pepper (paprika) as affected by technological and varietal factors. *Food Chem.*, **55**, 365–372.

Daood, H. G., Biacs, P. A. (2005): Simultaneous determination of Sudan dyes and carotenoids in red pepper and tomato products by HPLC. *J. Chromatogr. Sci.*, **43**, 461–465.

Márkus, F., Daood, G. H., Kapitány, J., Biacs, P. A. (1999): Change in the carotenoid and antioxidant content of spice red pepper (paprika) as a function of ripening and some technological factors. *J. Agric. Food Chem.*, **47**, 100–107.

Márkus, F., Kapitány, J. (2001): *A fűszerpaprika termesztése és feldolgozása.* (Production and processing of spice pepper.) Mezőgazdasági Szaktudás Kiadó, Budapest.

Somos, A. (1984): *The Paprika.* Akadémiai Kiadó, Budapest.

Speek, A. T., Schrijver, F., Shreurs, H. P. (1985): Vitamin E composition of some oils as determined by HPLC with fluorometric detection. *J. Food. Sci.*, **50**, 121–122.

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MICROSATELLITE MARKERS AND AUTOMATED FRAGMENT ANALYSIS TECHNIQUES FOR EFFICIENT AND PRECISE HYBRID IDENTIFICATION AND GENETIC PURITY TESTING IN PEPPER (*CAPSICUM ANNUUM* L.)

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As locus-specific co-dominant PCR-based markers that allow semi-automated, high-throughput investigation technologies, microsatellites are ideal tools for genotype identification. Eleven of a set of 114 microsatellite markers available at the Agricultural Biotechnology Center proved to be suitable to distinguish between the parents of at least one of nine sweet pepper hybrid combinations. Markers with the highest information capacity were found to be capable of distinguishing between the parents of four different hybrid combinations and exhibited up to four different alleles in 18 haplotypes.

Key words: pepper, hybrid identification, purity testing, microsatellites

Introduction

Pepper is one of the most important vegetable species in Hungary and worldwide. Seed production is one of the key factors in the Hungarian pepper industry. Red paprika powder, a typical, traditionally important export commodity in Hungary, is produced entirely from region-specific spice pepper varieties. The large number of sweet pepper varieties of special Hungarian types released during recent decades also represent great economic value. As in other vegetables, there is a growing trend towards the use of hybrid varieties of pepper (especially sweet pepper). By exploiting the heterosis effect in the F_1 generation, hybrids offer not only increased performance and uniformity, but also wider adaptability and reliability. Additional benefits of the commercialization of hybrids are the better protection of intellectual rights and the guaranteed annual seed sales to producers.

However, there is a need to be able to assess the hybrid (genetic) purity of seed lots, as part of the seed certification process and as a means of general quality control. Furthermore, hybrids, like all new varieties, must possess demonstrable distinctness, uniformity and stability (DUS).

In Hungary phenotypical characters, such as anthocyanin content or resistance against Tobacco Mosaic Virus (TMV), are presently employed predominantly for the identification of hybrids and for the quality testing of hybrid seed lots. However, the use of such markers restricts the breeder's latitude in designing hybrid combinations, as only parental genotypes with given phenotypic characters can be considered.

A special problem in pepper genome analysis is that cultivated varieties show an extremely low level of polymorphism when conventional genotyping systems such as isoenzymes or RFLPs are used. As locus-specific, co-dominant PCR-based markers that allow semi-automated, high-throughput investigation technologies, microsatellites are ideal tools for genotype identification. In contrast to conventional molecular markers, microsatellites show relatively high allelic variations in pepper. Similarly to other molecular markers, microsatellite polymorphisms are not dependent on environmental factors and can be investigated even in early phenophases.

The most crucial task in microsatellite-based hybrid identification is to obtain markers that exhibit allelic differences between the parental genotypes of a given hybrid combination.

Materials and methods

Plant material

For the hybrid identification experiments nine hybrid combinations were included from the sweet pepper breeding programme of the Vegetable Crops Research Institute: Ciklon F_1; Cecil F_1; Novarez F_1; Verde F_1; Apolló F_1; Century F_1; AK776 F_1; DH108×DH105 F_1 and DH99×DH105 F_1. For each combination at least three plants were selected from the paternal, maternal and F_1 lines. DNA was isolated from young seedlings according to Dellaporta et al. (1983) with slight modifications.

Microsatellite analysis

Microsatellite markers were developed from short insert genomic libraries of the pepper varieties Fehérözön or Blondy. PCR amplifications were carried out under standard conditions. Typically, after an initial denaturation step at 94°C for 3 min, 45 cycles were applied with the following parameters: 92°C for 1 min, 60°C for 1 min and 72°C for 1 min. The reactions were terminated by a final extension step at 72°C for 7 min. Forward primers were labelled with Cy5 at their 5' end to enable analysis on ALFexpressII automated laser fluorescence sequencers (Amersham-Pharmacia Biotech). Fragment sizes were calculated using the computer program ALFWin Fragment Analyzer (ver. 1.02, Amersham-Pharmacia Biotech) using Cy5-labelled internal and external lane standards.

Results

Eleven of the 114 microsatellite markers available at the Agricultural Biotechnology Center proved to be suitable to distinguish between the parents of at least one of the nine hybrid combinations investigated (Table 1). The markers with the highest information capacity could be applied for more than one hybrid combination: GPMS15 and GPMS112 distinguished between the parents of four different hybrid combinations, while GPMS8, GPMS37 and GPMS113 were able to identify three different hybrid combinations. Three further microsatellite markers proved to be suitable for two different hybrid combinations. The remaining three microsatellite markers showed allelic differences for one of the nine hybrid combinations.

Table 1

Microsatellite markers exhibiting polymorphisms between parental and F_1 genotypes of pepper hybrid combinations

Markers	Hybrid combinations		
	Ciklon ♂	Ciklon ♀	Ciklon F_1
GPMS8	178	193	178, 193
GPMS37	184	180	184, 180
GPMS113	128, 140	128, 148	128, 140, 148
GPMS159	287	290	287, 290
	Cecil ♂	Cecil ♀	Cecil F_1
GPMS8	195	184	195, 184
GPMS15	112	93	112, 93
GPMS37	178	180	178, 180
GPMS112	265	267	265, 267
GPMS159	304	290	304, 290
GPMS161	251	254	251, 254
	Novarez ♂	Novarez ♀	Novarez F_1
GPMS37	184	180	184, 180
CA1-19	138	168	138, 168
	Verde ♂	Verde ♀	Verde F_1
GPMS15	112	100	112, 100
GPMS29	260	262	260, 262
GPMS37	178	182	178, 182
GPMS112	265	277	265, 277
GPMS113	128, 149	128, 168	128, 149, 168
GPMS161	247	259	247, 259
	Apolló ♂	Apolló ♀	Apolló F_1
GPMS15	112	93	93, 112
GPMS113	128, 149	128, 168	128, 149, 168
	Century ♂	Century ♀	Century F_1
GPMS15	93	112	93, 112
GPMS93	251	235	251, 235
GPMS154	173	175	173, 175
	AK776 ♂	AK776 ♀	AK776 F_1
GPMS15	93	100	93, 100
	DH 108 ♂	DH 105 ♀	DH 108 × 105 F_1
GPMS8	195	184	195, 184
GPMS112	245	267	245, 267
CA1-19♂	138	168	138, 168
	DH 99 ♂	DH 105 ♀	DH99 × 105F_1
GPMS112	243	267	243, 267

The table shows allele sizes in base pairs (bp) calculated by the ALFWin Fragment Analyzer software after running on an ALFexpressII sequencer

To test the reliability of the microsatellite-based hybrid identification technology, 96 F_1 individuals of the hybrid *Cecil* were screened for heterozygosity with all the six microsatellite markers applicable for this hybrid. Individual F_1 plants were grown in the greenhouse and tested for TMV resistance, the genetic marker conventionally used for testing hybridity for this hybrid. The heterozygosity test gave consistent results for five microsatellite markers, which were in full concordance with the TMV results (data not shown).

On the other hand, in the case of marker GPMS112 an atypical paternal allele was found in about 20% of the F_1 plants (Fig. 1A). This indicated that these plants did not originate from the self-pollination of the maternal plant, but were hybrids and that the paternal lines probably had some extent of genetic impurity. This was indeed confirmed by a careful inspection of the paternal population of the cross in question. Pungent fruits were found in about 20% of the plants in the paternal population, while the original paternal genotype had non-pungent character. When 15 pungent and 15 non-pungent plants were selected from the paternal population, only the pungent individuals exhibited the atypical (shorter) allele, while all the non-pungent individuals showed the authentic paternal allele (Fig. 1B).

Discussion

Microsatellite markers have been successfully applied for cultivar and hybrid identification in a number of plant species (Becher et al., 2000; Esselink et al., 2003; Gémes Juhász et al., 2002; Tessier et al., 1999; Vosman et al., 2001; Yashitola et al., 2002). In pepper, hybrid testing based on microsatellites gave consistent, reproducible results that were in close agreement with the hybrid tests produced by scoring seedlings for anthocyanin or TMV markers. Though the ratio of usable microsatellite markers is relatively low in pepper (about 10% of the microsatellite markers used for this study), they are cost-effective and versatile. Microsatellite markers with high information content can be used for the identification of several genotypes and hybrid combinations. The most informative markers exhibited up to four different alleles for the 18 haplotypes included in this study. In the ideal case, 2 to 3 highly polymorphic markers should be available for each of the 12 chromosomes of pepper, to be able to fulfil all practical genotype identification tasks with a high level of probability. As the number of available microsatellite markers with known map positions has been continuously increasing in recent years (Lee et al., 2004; Sasvári et al., 2004) the availability of such marker sets can be foreseen in a relatively short time. Automated sizing technologies permit the genotyping of several hundred samples a day in a routine laboratory and can differentiate between alleles even if the size differences are as low as 2 base pairs. Additionally, microsatellite polymorphisms can be checked on DNA samples isolated from dry or germinated seeds, reducing the time and costs required for field or greenhouse tests.

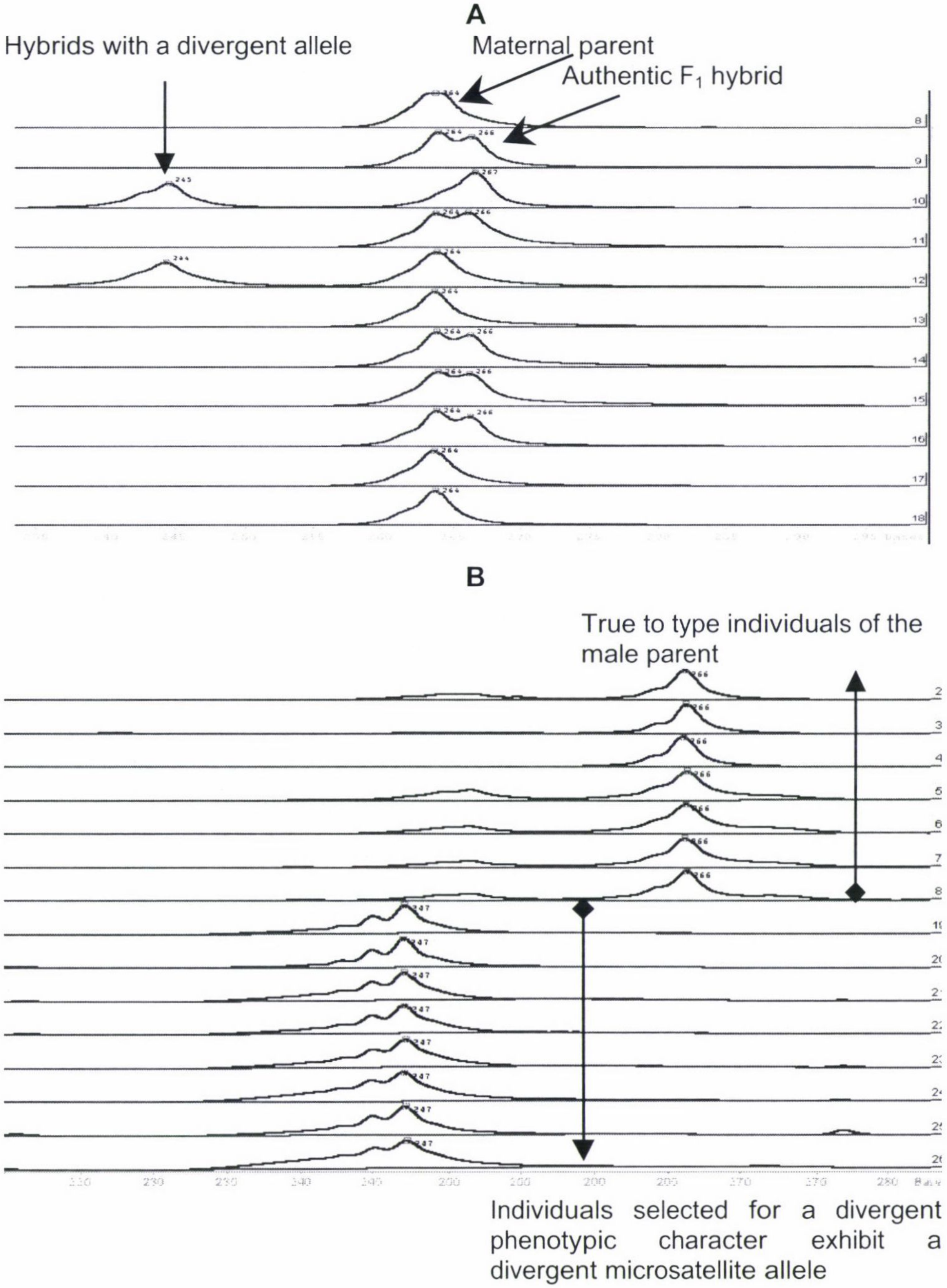

Fig. 1. (A, B) Checking genetic purity using microsatellite markers: Microsatellites may indicate heterogeneity that is not usually detectable by phenotypic selection. Chromatogram peaks represent microsatellite alleles detected by running on an ALFexpressII sequencer. For explanation see text

After adapting the technology for microsatellite-based hybrid identification, the same markers and techniques can be efficiently applied for different aspects of a pepper breeding programme, such as testing the genetic purity of breeding lines, the homozygosity of doubled haploids or the quality of hybrid seed lots obtained from different growers.

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References

Becher, S. A., Steinmetz, K., Weising, K., Boury, S., Peltier, D., Renou, J-P., Kahl, G., Wolff, K. (2000): Microsatellites for cultivar identification in *Pelargonium*. *Theor. Appl. Genet.*, **101**, 643–651.

Dellaporta, S. L., Wood, J., Hick, J. B. (1983): A plant DNA minipreparation: Version II. *Plant Mol. Biol. Rep.*, **1**, 19–21.

Esselink, G. D., Smulders, M. J. M., Vosman, B. (2003): Identification of cut rose (*Rosa hybrida*) and rootstock varieties using robust sequence tagged microsatellite site markers. *Theor. Appl. Genet.*, **106**, 277–286.

Gémes Juhász, A., Nagy, I., Zatykó, L. (2002): Using RAPD and microsatellite markers for pepper variety identification and variety protection. *Proc. Int. Conf. on Vegetables*, Bangalore, India, November 11–14, 2002, pp. 34–36.

Lee, J. M., Nahm, S. H., Kim, Y. M., Kim, B. D. (2004): Characterization and molecular genetic mapping of microsatellite loci in pepper. *Theor. Appl. Genet.*, **108**, 619–627.

Sasvári, Z., Bárdos, G., Ács, S., Stágel, A., Nagy, I. (2004): Construction of a new interspecific genetic map in pepper based on AFLP and microsatellite markers. *Proc. XIIth EUCARPIA Meeting on Genetics and Breeding of Capsicum and Eggplant*. Noordwijkerhout, The Netherlands, 17–19 May 2004. pp. 227–231.

Tessier, C., David, J., This, P., Boursiquot, J. M., Charrier, A. (1999): Optimization of the choice of molecular markers for varietal identification in *Vitis vinifera* L. *Theor. Appl. Genet.*, **98,** 171–177.

Vosman, B., Cooke, R. J., Ganal, M., Peeters, R., Isaac, P., Bredemeijer, G. (2001): Standardization and application of microsatellite markers for variety identification in tomato and wheat. In: Proc. Int. Symp. on Molecular Markers for Characterizing Genotypes and Identifying Cultivars in Horticulture. *Acta Hort.,* **546**, 307–316.

Yashitola, J., Thirumurugan, T., Sundaram, R. M., Naseerullah, M. K., Ramesha, M. S., Sarma, N., Sonti, R. V. (2002): Assessment of purity of rice hybrids using microsatellite and STS markers. *Crop Sci.,* **42,** 1369–1373

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Short communication

GENETIC TRANSFORMATION AND SHOOT REGENERATION PROCEDURE FOR PEPPER (*CAPSICUM ANNUUM* L.)

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In this short note a protocol was summarized based on almost a decade of experience on the regeneration and transformation of pepper. The recipe presented could be used efficiently in the genetic engineering of pepper. The essence of the regeneration and transformation of pepper is derived from detailed descriptions previously published by the authors on how the method was developed.

Key words: pepper regeneration, stable transformation, recalcitrant species, methodology

Regarding its suitability for *in vitro* regeneration and genetic transformation, pepper is considered to be recalcitrant.

A "shooter" mutant-based protocol for the genetic transformation and subsequent plant regeneration of pepper was described earlier (Mihálka et al., 2003). The novel transformation system was tested not only on pepper, but also on tobacco, muskmelon, potato and tomato, and was found to work efficiently for these species as well. Based on the results the "shooter" mutant-based transformation system seems to be applicable on a wide range of species without any change in the transformation and regeneration protocol. This transformation system is unique, as there is no need for preliminary tests or an efficient shoot regeneration system as a pre-requisite of genetic transformation. Furthermore, an important characteristic of the "shooter" mutant-based transformation system is that neither exogenous growth regulators nor selective agents such as antibiotics or herbicides are used during the procedure. The simple, universal character of the "shooter" mutant-based transformation system represents a potentially useful alternative for the genetic transformation of recalcitrant species. This short communication provides a brief description of the "shooter" mutant-based genetic transformation of pepper. More detailed information on the subject was reported by Mihálka et al. (2000; 2003).

Plant material

Cotyledons from 16–24-day-old aseptic seedlings of the Hungarian sweet pepper cultivar Fehérözön were used in the transformation experiments.

To produce aseptic plant material, seeds were surface sterilized with a 10 % $CaCl_2O_2$ solution supplemented with a few drops of Tween-20. After rinsing several times in sterile distilled water, the seeds were placed for germination into 1000 ml jars containing 100 ml MS basal medium, solidified with 0.7% agar and incubated in a culture room at 25°C in diffuse light.

Bacterial strains

The "shooter" mutant strains ShooterG Rif^R and pGV3170 RifR^R, carrying the binary plasmids pRGG hpt and pRGG neo, were used in the transformation experiments (Mihálka et al., 2000; 2003). Binary plasmid vectors were introduced into pGV3170 Rif^R and ShooterG Rif^R by triparental mating (Ditta et al., 1980) using pRK2013 (Figurski and Helinski, 1979) as helper.

Co-culture of explants with Agrobacterium

Fresh over-night *Agrobacterium* cultures grown in liquid YEB medium were diluted the next morning to 1:10 with minimal AB medium (Chilton et al., 1974) containing 100 μM acetosyringone and grown to OD 0.6–0.8. Explants were co-cultivated with *Agrobacterium* for two days in liquid MSB5gl medium (MS salts, B5 vitamins containing 20 g/L glucose) supplemented with 100 μM acetosyringone.

Tissue culture and plant regeneration

After co-cultivation the explants were transferred to liquid MSB5gl medium supplemented with 500 mg/L cefotaxim and incubated for at least 4–6 hours in order to eliminate *Agrobacterium*. The explants were than transferred onto solid MSB5gl medium supplemented with 300 mg/L cefotaxim, and transferred to fresh medium at 2–3-week intervals. No exogenous growth regulators were applied during the entire tissue culture procedure after co-culture with wild-type and mutant *Agrobacterium* strains, except for the rooting of the regenerated pepper shoots, which was conducted on MS basal medium supplemented with 0.5 mg/L IAA, 15 mg/L glucose and 15 mg/L maltose. When *Agrobacterium* carrying the pRGG plasmid was used, co-transformants were selected on MS medium containing 25 mg/L hygromycin or 150 mg/L kanamycin. Plants rooted on selective medium were transferred to the greenhouse.

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References

Chilton, M. D., Currier, T. C., Farrand, S. K., Bendich, A. J., Gordon, M. P., Nester, E. W. (1974): *Agrobacterium* tumefaciens DNA and PS8 bacteriophage DNA not detected in crown gall tumors. *Proc. Nat. Acad. Sci. USA,* **71**, 3672–3676.

Ditta, G., Stanfield, S., Cobbin, D., Helinski, D. R. (1980): Broad host range DNA cloning system for Gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. *Proc. Nat. Acad. Sci. USA*, **77**, 7347–7351.

Figurski, D. H., Helinski, D. R. (1979): Replication of an origin-containing derivative of plasmid function provided in trans. *Proc. Nat. Acad. Sci. USA*, **76**, 1648–1652.

Mihálka, V., Fári, M., Szász, A., Balázs, E., Nagy, I. (2003): Optimized protocols for efficient plant regeneration and gene transfer in pepper (*Capsicum annuum* L.). *J. Plant Biotechnol.*, **2**, 143–149.

Mihálka, V., Balázs, E., Nagy, I. (2003): Binary transformation system based on shooter mutants of *Agrobacterium tumefaciens*: A simple, efficient and universal gene transfer technology allowing marker gene elimination. *Plant Cell Reports*, **21**, 778–784.

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Review

PEPPER TAXONOMY AND THE BOTANICAL DESCRIPTION OF THE SPECIES

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The *Capsicum* genus, which originates from the American continent, contains species with a chromosome number of n=12. The plants have white, lilac or purple flowers, and hollow fruit of very varied shape and size, containing glands alongside the veins that produce a pungent alkaloid known as capsaicin. The majority of varieties in the species *C. annuum*, grown in the largest volume throughout the world and consumed as fresh vegetables or ground spices, are non-pungent. Interspecific crosses are often possible between *C. annuum* and related, white-flowered species, thus facilitating breeding for resistance against various diseases and pests and the search for new, valuable traits. Species with lilac and purple flowers can be crossed with each other, but direct crosses with white-flowered species are unsuccessful.

Key words: pepper, *Capsicum*, wild species, interspecific hybrids, resistance, capsaicin

Introduction

Pepper was first introduced into Hungary from the American continent at the turn of the 18th century, probably through Spain and Portugal, but possibly also through Turkey and Bulgaria. After a few earlier mentions, the first competent description was given in the herbal published by Diószegi and Fazekas (1807), who used the nomenclature of Linnaeus to list two species of the *Capsicum* genus, *C. annuum* (paprika, Turkish pepper) and *C. sinense* (Chinese pepper). The fruit of both species was described as dry and hollow, and the description of the Chinese variety conforms perfectly to the appearance of the *C. chinense* species (shrubby stem, paired peduncles, oval drooping fruit, hairy peduncles, yellow fruit). It is highly probable that the authors had seen both species, as Linnaeus's work only listed the *C. annuum, C. frutescens, C. baccatum* and *C. grossum* species. *C. grossum* is a large-fruited variant of *C. annuum* and is considered to be a synonymous term. The species *C. chinense* was not mentioned by Linnaeus, and was not described until later, in 1767, by Jacquin. The fifth cultivated species, *C. pubescens*, was described in 1799 by Ruiz and Pavon. These five species are still grown, wherever the ecological conditions are satisfactory, but only *C. annuum* is produced in large quantities, including varieties with an enormous range of fruit size, shape and colour. The Habanero variety of *C. chinense* and the Tabasco variety of *C. frutescens* are also known and consumed throughout the world, but the varieties Aji (*C. baccatum*) and Rocoto (*C. pubescens*) are only grown on a small scale, primarily in tropical countries with a hot climate, and the fruit of these species can be found in local markets in areas where they are indigenous.

All the species in the *Capsicum* genus originate from the American continent. The gene centre is thought by McLeod et al. (1982) to be the central region of Bolivia, a semi-arid area, free of frost, at a height of 2000 m above sea level. An ancestral species similar to the existing species *C. chacoense* is assumed to have spread to other parts of the continent from this nuclear area and to have given rise to the *Capsicum* species cultivated today through a series of mutations and spontaneous hybridisations. Wild species of this genus are found in the northern part of Argentina, in Bolivia, throughout Brazil, in Peru, Columbia and Venezuela, in all the Central American countries, in Mexico and even in the southern part of the USA. Pickersgill (1982), on the other hand, places the gene centre in the hilly region of Brazil, since the largest number of species originate from this area. All the species in the genus are still not known, since botanists, particularly from abroad, are unable to explore the areas richest in species due to the critical standard of public security, but there are thought to be some 25–30 species in all. The taxa included in the *Capsicum* genus have 12 chromosomes and produce hollow fruit containing the pungent substance capsaicin.

Numerous archaeological finds confirm that the consumption and cultivation of various pepper species began at least 7000 years ago. An analysis of the finds indicates that they were cultivated, since they contain traits unknown in wild species (short calyx teeth, yellow fruits that do not soften). Birds played a key role in the spread of the tiny-fruited, pungent ancestral species, i.e. in the evolution of the species, since, unlike mammals, they are not affected by their pungency.

Among the species in the *Capsicum* genus, genetic relationships are clearly indicated by the flower colour, so this is used as the basis for taxonomical classification. In addition to the white- and purple-flowered taxonomical groups, it is worth distinguishing a further group that is intermediate between these two and plays an important connecting role.

– The white-flowered group includes the most widely cultivated species, *C. annuum* var. *annuum* and it wild form, *C. annuum* var. *glabriusculum*, the species *C. chinense* and *C. frutescens*, which are also cultivated, and two species which are similar to the "ancestral species": *C. chacoense*, with thin branches and small, soft fruit, and *C. galapagoense*, with slightly hairy leaves and stem and tiny, soft fruit.

– The intermediate group includes *C. baccatum* var. *pendulum*, a cultivated species whose white flowers have a yellow spot at the base, its wild form, *C.*

With the help of this descriptive method, the characteristics of the California Wonder variety, taken in the genetic sense as the "wild species", can be described as follows, with the characteristics of the best-known varieties differing from this "wild" form in brackets:

– the stem below the cotyledons is purplish-green (completely purple, green or anthocyanin-free),
– the main axis branches after 10–12 nodes (15–30 nodes),
– the main axis exhibits continuous (determinate, fastigiate) growth,
– the main axis and the side-shoots are glabrous (hairy),
– the nodes are purplish-green (purple, completely green, anthocyanin-less),
– the leaves are entire (slightly sinuate),
– the leaves are glabrous (hairy),
– the leaves are dark green (mid-green, light green, yellow, purple),
– the leaf-blade has broad (narrow) shoulders,
– the leaf tip is rounded (pointed),
– the calyx is white (purple),
– the flowers are arranged singly (in pairs, in bunches) at each node,
– the flower stalk droops (is erect, semi-erect),
– the anther is pale purple (dark purple, yellow, anthocyanin-less),
– the style and the filament are greenish-white (white, purple),
– the fruit shape is blocky (spherical, flattened sphere, conical, long and pointed),
– there are four (two to ten) veins in the fruit,
– the veins are non-pungent (pungent),
– the fruit flesh is dark green (white, light green, mid-green, purple) when market-ready,
– the fruit flesh is red (white, yellow, orange, pink, green, brown, black) when biologically ripe,
– the flesh is hard (soft),

– the plant gives a susceptible response to Tobamo viruses (*Tobacco mosaic virus* - TMV, *Tomato mosaic virus* - ToMV, *Pepper mild mottle virus* - PMMoV), the leaves and fruit are deformed and exhibit mosaic symptoms (plants containing the *L1, L3* or *L4* alleles of the *L* gene respond with cell death or local necrosis to artificial inoculation of the cotyledons and leaves with Tobamo viruses, the fruit remains symptom-free),

– the plant gives a susceptible response to Tospo viruses (*Tomato spotted wilt virus* - TSWV), the leaves and fruit are deformed and exhibit mosaic symptoms (the cotyledons and leaves of plants containing the *Tsw* gene respond to artificial inoculation with cell death or local necrosis, the fruit remains symptom-free),

– in response to infection with the bacterium *Xanthomonas vesicatoria* water-soaked spots appear on the leaves, which then wither and die; brown water-soaked spots causing blisters on the epidermis appear on the fruit, after which the epidermis parts from the tissues below, bursts, and dries with white coloration (in response to natural infection on plants carrying the *Bs-2* gene, irregularly shaped lesions 2–3 mm in diameter, that turn red and then wither, appear on the leaves, but the fruit is symptom-free; on the leaves of plants carrying the *gds* gene, a few cells localise the pathogen by cell enlargement and possibly cell division),

– the plants are susceptible to nematodes (plants containing the *N* or *Me* genes are resistant).

Naturally this list could be extended to include other traits.

One of the most important characteristics of pepper is the pungency caused by the capsaicin content, which is produced in glands in the veins. The presence or absence of capsaicin is controlled by a single dominant gene (*C*). Without exception the wild species are all extremely pungent, and among the five cultivated species only a few varieties of the *C. annuum* species are non-pungent. Birds probably played an important role in spreading the species in the *Capsicum* genus because, unlike mammals, they are not affected by the pungency of the capsaicin. Among the mammals, guinea pigs are the most sensitive and rabbits the least. It is difficult to measure pungency. One method is the scale elaborated by Scoville (1912), who diluted one part of pungent material with a million drops of water and tested it organoleptically. Scoville's Heat Units (SHU) can be converted to ppm using the formula 15 SHU = 1 ppm capsaicin.

A number of well-known varieties have the following SHU values: among the *C. annuum* varieties, California Wonder: 0, Ancho: 2000, Jalapeno: 3000–8000; the *C. frutescens* variety Tabasco: 30,000–50,000; the *C. chinense* varieties Habanero: 100,000–300,000 and the hottest, Red Savina Habanero: 350,000–577,000, while pure capsaicin has a value of 15–16 million. The Hungarian standard determines the pungency of spice paprika using spectrophotometric or HPLC methods and expresses the value in mg/kg. Chemists use the term capsaicinoid, the main components of which are capsaicin, dihydrocapsaicin and nordihydrocapsaicin. The concentrated capsaicinoid used by the pharmaceutical industry is known as Oleoresin Capsicum.

In describing varieties, serious problems are caused by confusion in the use of taxonomical terms (DeWitt and Gerlach, 1990; DeWitt and Bosland, 1996). In Hungarian the word paprika is used for both vegetable peppers and spice paprika, whereas abroad the term is generally used only for the spice. Unfortunately, even respected textbooks use the term "Hungarian paprika" for both the white-fleshed, sweet, elongated cone-shaped vegetable peppers of the Cecei type and the sweet, pointed spice paprika types that have green flesh, turning red when ripe. This confusion is probably also true of the many types of famous peppers grown in other countries and suited for various end uses. Nevertheless, an attempt will be made below to describe the major types of *Capsicum* cultivated in various parts of the world, including some of the better known variety names. The varieties have been grouped according to the shape of the fruit.

Blocky or Bell types

These sweet peppers, which have large, sweet fruit with thick flesh, suitable for both fresh consumption and processing, are perhaps the best known and most widely cultivated. The most typical variety in this group is California Wonder, which has been known since the 1850s, but whose origin is likely to remain a mystery. The basic type turns from green to red as it ripens, but orange, yellow and brown varieties are now available, while some stay green when ripe and others ripen from purple to red. The old Italian Quadrato d'Asti types, which are yellow or possibly red when ripe, are somewhat bigger with more pronounced corrugation, but may be slightly deformed in shape. These types characteristically have a small number of capsaicin-producing glands along the broad, white veins, making the fruit slightly pungent. The popular Lamuyo type, developed by French breeders from a cross between the short blocky (Bell type) variety Yolo Wonder and the long, pointed French variety Lamu, is now considered to be a separate category. Other blocky types are the Austrian variety Neusiedler Ideal and the thin-fleshed variety Dolma, used in Turkey to make stuffed peppers. The extremely early, high-yielding Ace varieties, mainly familiar on the Japanese market, have medium-sized blocky fruit with thin flesh. For a long time there were no blocky peppers with a completely white fruit colour. The Blondy variety and other white-fruited (ivory) varieties were designed by a number of breeding companies primarily for the Hungarian market, but consumers still prefer the traditional long conical shape of the Cecei type.

Tomato-shaped varieties

The corrugated Marmand (Fóti) type of tomato which gave its name to this group is no longer widely known. Tomato-shaped pepper varieties are grown in a relatively limited region, primarily for Hungarian and Romanian (Gogosar) markets, though it is sometimes sold in Bulgaria, Serbia, Turkey, Italy, Israel, and even in the USA. Due to its excellent flavour, large quantities are processed by the frozen foods industry, as well as being sold for fresh consumption.

Conical and round forms

The conical Italian form Cuneo, which is slightly pungent, turns yellow or possibly red when ripe, and has excellent flavour. The French variety Antibois (and the Hungarian version Szarvasi 11 selected from it), and the American heart-shaped varieties Truhart Perfectin and Pimiento are popular with the processing industry for their thick flesh. The round, white-fleshed apple pepper type, mainly used for pickling, is mainly popular in Hungary, but can also be obtained in neighbouring countries and even in America. The cherry types (spherical or slightly conical), which come in various sizes, most of them pungent, are mainly used for pickling, but are also consumed in dried form.

They may also be used for decoration. The old Mexican variety Cascabel was also cherry-shaped, but had very long peduncles. The famous Spanish variety Bola, a slightly conical sphere with a tendency to be corrugated, is grown as a spice. The name Bola comes from the weapon developed by the American Indians, which consisted of two balls attached to a long rope. Varieties with tiny spherical or conical fruit and determinate growth are popular as ornamentals.

Elongated conical type

The elongated conical type, which consists of a large number of varieties, is the most familiar type of pepper for fresh consumption in Hungary and in the surrounding countries of Eastern Europe. One group consists of the Cecei type, new variants of which have larger, more elongated fruit and are grown mainly in the field. The old Cecei landrace and types similar to it were originally pungent, but due to changes in eating habits and the demands of the export market, the first large-scale pepper breeding programme set up in the 1950s led to the development of the non-pungent varieties Cecei Édes and Javított Cecei, though the pungent versions are still grown in private gardens (Bogyesz, Újmajori). The slightly more elongated HRF hybrid variety has been classified as a new category over the last 20 years and all the seed companies endeavour to produce novelties for this market. Varieties with light green fruit having a pleasantly pungent flavour are popular in Slovakia (PCR), while sweet varieties, again with light green fruit, are sold in Romania, Serbia, Croatia, Poland and in ex-Soviet countries. Numerous variants of these light or dark green types are also popular on the American continent. Ancient Mexican varieties (Ancho, Poblano) can also be listed here, but only on the basis of fruit shape and size. Both varieties develop tall, robust bushes on which the long-stalked fruit ripen from dark green to brownish-red or even true brown (Mulato) or black (Negro). The thin-fleshed fruit, which are used fresh for cooking or are sold dried, are available throughout Central America.

Corno type

The Corno (horn-shaped) varieties, familiar primarily in the Mediterranean region, have slightly corrugated fruit, 18–20 cm in length and 4–6 cm wide at the shoulders, which are sometimes crumpled. The fruit ripen from dark green to red or yellow and are mostly sweet, though there are also a few pungent varieties. They are produced almost exclusively for the processing industry, though they are also suitable for fresh consumption when ripe (Dolce Italiano). There is considerable overlapping between the Corno and Kapia types, especially in recently bred hybrids. The old, fragrant, light-green types Cuban, Biscayne and Aconcagua are popular in America for salads or cooking. The Italian variety Corno Mantovano is a similar type.

Kapia type

The Kapia varieties are the best-known type in the Balkans and have become extremely popular in recent years, probably due to their introduction into Western Europe by guest workers. They are also in demand for the processing industry, as they do not rot during transport. The skin is very thick, but can be pulled off after roasting, while the thick flesh is delicious and easily digestible.

Banana type

The Banana type familiar on American markets consists of pepper varieties with long, pointed, light green fruit. Depending on the fruit length and shoulder width, and on the smoothness, corrugation or crumpling of the skin, numerous types can be distinguished, one or other of which is grown in almost all regions where pepper is regularly consumed. Flavours range from mild to extremely pungent. These types (Hegyes erős) were previously sought by Hungarian consumers in the winter season, but now that other types with larger fruit are available throughout the year their consumption has somewhat decreased. In addition to fresh consumption, they are also popular with the processing industry, especially on the American continent. The varieties Sigaretta, Lombardo and Spirál, which have extremely narrow, crumpled fruit, light green in colour and either slightly pungent or sweet, are only used for pickling. The mid-green, slightly pungent Demre variety, an essential condiment in the Turkish cuisine, is grown in enormous quantities and consumed mainly roasted.

Jalapeno type

Jalapeno, one of the best-known types in Mexico (named after the town of Xalapa), has thick pungent flesh, turning red, brown or even yellow when ripe. The skin is neither corrugated nor crumpled, but tends to crack along the length of the fruit. The extent of cracking varies considerably and makes the fruit very decorative. The fruit size is varied, but the basic type is 6–10 cm in length and 2–3 cm in diameter, being the same width at the shoulder and tip. This type is used fresh or conserved for the famous sauce salsa, but is also stored dried or smoked. The best salsa can be made from freshly picked peppers of the Serrano variety, also an old Mexican variety. The fruit of the Serrano type softens when ripe due to the dominant S gene, so it is difficult to store.

Chili type

Peppers of the Chili type are grown all over the world. The fruit may be short (5–6 cm) or long (15–18 cm), the shoulder width is 1–3 cm, the skin is generally smooth, though it may be slightly crumpled or corrugated, and the fruit may be curved. The chief characteristic of these varieties is their thin flesh, which dries easily, making them easy to store dried, though they are sometimes used fresh. The majority of varieties are pungent, though numerous sweet varieties are also known.

Hungarian spice paprika and Cayenne types

This group includes the famous Hungarian spice paprika (more than 20 varieties registered by breeders from the Kalocsa and Szeged institutes), the similarly sized varieties Papri King and Papri Queen, the American variety Anaheim, which has somewhat larger fruit, sweet types from New Mexico, the ancient Mexican variety Pasilla, consumed after drying, the pungent, narrow-fruited Korean varieties, and the Cayenne types grown throughout the world, which could also be considered to be a distinct group. Pungent, small-fruited varieties are a basic ingredient, fresh, dried or ground, of the exotic cuisines of Asian and African countries.

Capsicum annuum* L. var. *glabriusculum

The small (1–2 cm) spherical or slightly conical, pungent fruit of the wild ancestor of the cultivated species, which still grows in the wild in Mexico, is becoming increasingly popular. Attempts to cultivate the Piquin and Chiltepin types, which soften when ripe, are now in progress in Mexico, but it is difficult to ensure the semi-shaded, humid atmosphere of the original growing site.

***Capsicum chinense* Jacq.**

After the *C. annuum* species, varieties of this species are probably the most widely cultivated. The forty or so variants described by Barnabe Cobo, the naturalist who was a contemporary of Columbus, probably belonged to this species. Enormous variability was observed in fruit size, ranging from wheat grain to large plum size, and the fruit colour also varied greatly. Growth habit ranged from prostrate to erect, and the leaves and stems from completely glabrous to very hairy. Most variants of this species originate from the Amazon Basin and the Caribbean region, but indigenous variants are also found in Central America and Mexico. The ancestor of the *annuum–chinense–frutescens* species complex probably arose somewhere in South America (possibly in Columbia). The domestication of the three species probably started from a common ancestor, giving rise to *C. annuum* in Mexico, *C. frutescens* in Central America and *C. chinense* in the Amazon Basin. One theory considers *C. chinense* to be a cultivated variant of *C. frutescens*, but if large numbers of plants of both species are examined in various phenological phases (from the seedling stage to full maturity) taxonomical determination can prove extremely difficult. The frequently mentioned difference in the colour of the corolla (white in *C. chinense* and green in *C. frutescens*) may be used for classification, but if the length of the stem below the cotyledons is taken as the basis, numerous lots identified as *C. frutescens* or *C. chinense* prove to have a short stem, pronouncing them to be of the wild type, and *vice versa*. The *frutescens* form and all other wild *Capsicum* species are characterised by erect peduncles, but even this cannot be generalised. The number of fruit per node is also an unsuitable trait for classification, as types with one or as many as 4–6 fruit per node can be found in both groups. Fruit that soften when ripe (*S* gene) occur in both the wild and the cultivated types. The best-known variety of the *C. chinense* species is Habanero (from Havanna), a pungent variety which is grown in large quantities on the Yucatan Peninsula in Mexico, but is also cultivated in many Central American countries and is becoming increasingly popular with private gardeners. Another well-known *C. chinense* variety is Scotch Bonnet, grown chiefly in Jamaica, but also popular with gardeners. All the *C. chinense* types are pungent, but Habanero tops the list, with an SHV value of 300–500 thousand, making it 30–40 times more pungent than the Jalapeno type, which is also extremely pungent. After the colonisation of America it was various types of this species that spread to African and Asian colonies. The fruit are added to fresh salads, but their main use is to produce very hot sauces. They are sold dried and ground, and it is wise to wear a mask when handling them.

Most *C. chinense* types can be crossed with the *C. annuum* species to produce fertile progeny, and they are an important basic material for resistance breeding. The *L3* gene for resistance to an aggressive strain of Tobamo viruses (TMV, ToMV, PMMV) was found in samples of this species, as was the *Tsw* gene for resistance to Tospo viruses (TSWV). Some lots are extremely susceptible to nematodes, but resistant types are also known.

Capsicum frutescens L.

This third member of the *chinense–annuum–frutescens* species complex carries the largest number of wild traits. It is found on much the same area as the *C. chinense* species, i.e. throughout Central America and in the northern part of South America. It can be crossed with both the other species, though the fertility of the F_1 hybrids is sometimes only 5–10%. In the F_2 generation and among backcross progeny, however, completely fertile plants are often found. If only the wild traits are considered, this species is not as variable as *C. chinense*. The best-known variety is Tabasco (named after a Mexican town), used in making the famous hot sauce. In the 1840s the cultivation of this variety was begun in the state of Louisiana and the maintenance of the Tabasco variety, now a registered trade mark, is carried out by the McIllheny family. The majority of the commodity production, the extremely labour-intensive manual picking of the perishable fruit and its rapid salting (conservation) are carried out in poorer countries in Central and South America, after which the salted product can be stored for years in wooden barrels prior to processing. In the seedling stage Tabasco plants are stocky, but they grow into large, decorative bushes. At each node there are generally two light green flowers, on erect stalks, followed by greenish white fruit, which are 4–5 cm in length with a shoulder width of 1 cm and turn light red when ripe. Types similar to Tabasco are cultivated all over the world. In the Amazon Valley the variety Malagueta is the most popular, and variants of this variety are also grown in Africa and Asia. Like *C. chinense*, *C. frutescens* is also pungent, but with a maximum value of 30–50 thousand SHV. Many varieties in the species contain genes for resistance to Tobamo and Tospo viruses, but breeders prefer to use the more readily crossable *C. chinense* species. *C. frutescens* is also an important source of resistance to Potyviruses (PVY, TEV), Cucumoviruses (CMV) and nematodes.

Capsicum chacoense Hunziker

This is a typically wild species, which is not cultivated and hardly consumed, due to its tiny fruit, which soften when ripe. It is only found in relatively dry areas of Argentina, Paraguay and Bolivia. It is easily recognisable and distinguishable from other species as it forms bushes with numerous thin branches, which turn purple and become woody, and has greyish-green, heart-shaped leaves. The shoot branches after the 15–20th node, and bears single flowers with snow-white corolla, yellow anthers and long calyx teeth, and elliptic fruit, measuring 1 cm at the shoulder and 2–3 cm in length, that soften when ripe. The flowers contain an unusually large drop of nectar at the base of the filaments. Although its phenotype differs from that of *C. annuum* and other white-flowered species, it can be crossed with *C. annuum* when used as the male parent. If *C. chacoense* is the female parent in the cross, the F_1 progeny have degenerate, sterile flowers, bearing practically no fruit. The *L4* gene for resistance against the most aggressive strain of the Tobamo viruses and the *Bs-2* gene responsible for resistance to the bacterium *Xanthomonas vesicatoria* were both found in samples of this species.

Capsicum galapagoense Heiser et Smith

This species is only found on the Galapagos Islands, which lie exactly on the Equator and have a unique climate. The fact that in Hungary it flowers in spring or autumn, but not in summer, is probably due to the daylength. The whole plant is hairy, from the cotyledon to the fruit stalk. No anthocyanin is found in any part of the plant. The flowers are white, the anthers are very pale yellow, and the very tiny (0.5 cm) fruit are pungent, have no calyx tooth and soften when ripe. This species is not used in breeding programmes, but it can be crossed with *C. annuum*.

Capsicum baccatum var. *pendulum* (Willd.) Eshbaugh

This species, which grows wild in South America, mainly in Bolivia and Peru, is now cultivated under the name Aji from Ecuador to Argentina, and even in the southern part of the USA and in India. It is the cultivated variant of *C. baccatum* var. *baccatum* (syn. var. *microcarpum*). In general it forms a large bush with thick branches, though plants with thin, prostrate branches have also been found. Its best-known trait is the greenish-yellow spot at the base of the white corolla, caused by the presence of a single dominant gene (yellow spot - *Ys*). It has an extremely long fruit peduncle, which causes the fruit to droop and resulted in its erroneous name, as the genotype is actually erect. The fruit vary greatly in size and shape, similarly to those of *C. chinense*. The largest fruit may have a shoulder width of 2 cm and a length of 12–15 cm. When market ready the fruit are pungent, though milder, almost sweet variants are also known. The fruit colour ranges from white to green, turning red, yellow or orange when ripe. The calyx teeth are moderately long. Some types are extremely daylength-sensitive and neither flower nor set fruit in winter, while others are not sensitive to the paucity of light in winter. It is difficult to produce hybrids between this species and *C. annuum*. *C. baccatum* var. *pendulum* × *C. annuum* combinations have poor fertility in the F_1, and in the F_2 many plants have male- and female-sterile flowers with a completely reduced corolla. The *Ys* gene responsible for the yellow spot is soon lost from the progeny, suggesting the loss of chromosome sections. The opposite combination tends to be more successful if the *C. annuum* variety belongs to the old Mexican group (Jalapeno, Serrano, Ancho) rather than to the European group. The *C. baccatum* var. *pendulum* species contains a large number of valuable resistance genes, but no data are available on their successful transfer. In the same way as the *Ys* gene, the traits desirable for breeding are probably absent from the progeny obtained after backcrossing due to the loss of chromosome sections. The species can be easily crossed in both directions with the *C. praetermissum* species.

***Capsicum baccatum* var. *baccatum* (Willd.) Eshbaugh**

This is the wild form of the cultivated species *C. baccatum* var. *pendulum*. The stem below the cotyledons is shorter and the cotyledons, leaves, flowers and fruit are smaller. There are one or two white flowers at each node, the petals of which are wider open and have a greenish-yellow spot (*Ys* gene). The pungent red fruit, which are 1.0 cm in diameter or possibly elongated to a length of 1.5–2.0 cm and soften when ripe, remain erect despite the long stalk, due to the small fruit weight. It is even more difficult to cross with *C. annuum* than the cultivated variant, but hybrids with good fertility in both the F_1 and F_2 generations can be obtained readily in both directions with *C. praetermissum*.

***Capsicum praetermissum* (Hunz.) Heiser et Smith**

This species is indigenous in the south east of Brazil and represents a transition between the purple- and white-flowered species. Like the wild types, the stem below the cotyledons is short, but the plants have a strong root system and may grow to a height of 1.0–1.5 m, sometimes producing strong side-shoots.

The whole plant, including the leaves, stem and peduncle, is slightly hairy. The petals have a dark or sometimes light purple edge, and a greenish-yellow spot, as in the *C. baccatum* species. The 2–3 flowers at each node have erect peduncles and wide open corollas, while the pungent fruit, which soften when ripe, are spherical or slightly oval, with a diameter of 0.5–1.0 cm, and have short calyx teeth. All the known types turn red when mature. The species can be easily crossed with *C. baccatum* and *C. eximium*, resulting in fertile progeny.

Capsicum eximium Hunziker

The growth type of this species is similar to that of *C. cardenasii* and *C. tovarii*, having a large number of thin branches, long lanceolate leaves with a slightly undulating surface and a low level of hairiness. The main axis only branches after the first 15–20 nodes, where 2–3 flowers develop, with long calyx teeth, wide open purple corollas and greenish-yellow spots. The tiny (0.5 cm) pungent fruit turn red and soften when ripe. This species is slightly daylength-sensitive, setting fruit more efficiently in late summer and autumn. It is indigenous in Bolivia and northern Argentina. It can be crossed easily with its close relatives, *C. cardenasii* and *C. tovarii*, with the transitional species *C. praetermissum* and with the brown-seeded species *C. pubescens*.

Capsicum cardenasii Heiser et Smith

The growth type is similar to that of *C. eximium*, producing a large number of side shoots after the main axis branches after the first 15–20 nodes. The leaves are flatter than those of *C. eximium*, with no hairs and a shiny surface. There are 1–3 purple campanulate flowers at each node, with long calyx teeth. The corolla has a whitish spot and the pungent fruit, 0.5 cm in diameter, turn red and soften when ripe. Seed setting is poor, probably due to daylength sensitivity and the auto-compatibility described by Yaqub and Smith (1971). It is only indigenous in Bolivia. It can be readily crossed with *C. eximium* and *C. pubescens* to give fertile progeny.

Capsicum tovarii Eshbaugh, Smith et Nickrent

This species, which is only indigenous in Peru, has a prostrate growth habit with many thin branches, similar to that of *C. eximium* and *C. cardenasii*, but the leaves are much narrower, with a glabrous, slightly blistered surface. The flowers are campanulate, but less open than those of *C. cardenasii*, with no spot and no calyx teeth. There are 3–5 flowers at each node and the pungent fruit are 0.5 cm in diameter, turning red and softening when ripe.

Capsicum pubescens Ruiz et Pavon

This exotic species has brown or brownish-black seeds and is cultivated under the names Rocoto, Locoto, Ilata, Chile manzano (apple-shaped), Chile perón (pear-shaped) and Chile caballo (horse pepper). Interestingly enough, there is no difference between the wild form and the cultivated types. No forms with tiny, softening fruit are known. The ripe fruit may be red, yellow or even brown, suggesting that it has been cultivated for a very long time, as confirmed by archaeological data going back six or seven thousand years. It must be consumed fresh or processed immediately, as its thick-fleshed, succulent fruit rot quickly and cannot be dried. The stems and leaves are hairy (giving it its name). The growth habit is prostrate, with long side-shoots forming after the first 10–15 nodes. A single, cup-shaped, purple flower with a white spot develops at each node, and the pungent fruit, 2–3 cm wide at the shoulder and 4–5 cm in length, ripen from dark green to red, yellow or brown. Under greenhouse conditions some plants set few fruits, probably due to climatic reasons or to the excessive nitrogen content of the soil, but auto-incompatibility may also be a problem, as described for *C. cardenasii*. Although its pungency is not excessively high according to the Scoville scale (30–50 thousand SHU), many people find it even hotter than the famous Habanero variety of *C. chinense*. This is a typical plant species of tropical mountains, cultivated in the South American Andes from Columbia to Chile and Mexico. This species has the best cold tolerance, which could make it a useful species for pepper breeding programmes. It can only be crossed with its close relatives, *C. cardenasii, C. tovarii* and *C. eximium*, but using *C. praetermissum* or *C. baccatum* as intermediate species, conventional crosses can be continued up to the cultivated *C. annuum* species. This process can be considerably accelerated by means of gene manipulation. This exotic species could also be of importance in breeding for resistance against common pathogens.

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References

DeWitt, D., Gerlach, N. (1990): *The Whole Chile Pepper Book*. Little, Brown and Company, Boston, 373 p.

DeWitt, D., Bosland, P. (1996): *Peppers of the World*, Ten Speed Press, Berkley, 219 p.

Diószegi, S., Fazekas, M. (1807): *Magyar füvészkönyv*. (Hungarian Herbal.) Nyomtatta Csáthy György Debreczenbenn, 177 p.

Eshbaugh, W. H. (1970): A biosystematic and evolutionary study of *Capsicum baccatum* (Solanaceae). *Brittonia*, **22,** 31–43.

Eshbaugh, W. H., Smith, P. G., Nickrent, D. (1983): *Capsicum tovarii* (Solanceae) a new species of pepper from Peru. *Brittonia,* **35,** 55–60.

Heiser, C. B., Smith, P. G. (1958): New species of Capsicum from South America. *Brittonia*, **10,** 194–201.

Hunziker, A. T. (1950): Estudios sobre Solanaceae. I. sinopsis de las especies silvestres de Capsicum de Argentina y Paraguay. *Darwiniana,* **9,** 225–247.

Jacquin, N. J. (1770–1776): *Hortus Botanicus Vindobonensis*. **1-3,** Viennae.

Linne, C. (1737): *Hortus Cliffortianus*. Amsterdam.

Linne, C. (1753): *Species Plantarum Holmiae.*

Scoville, W. L. (1912): Scovill organoleptic test. *The Journal of the American Pharmacists Association*. **1,** 453–454.

Ruiz, L. H., Pavon, J. (1794): *Flora Peruvianae et Chilensis prodromus*, Madrid.

Yaqub, C. M., Smith, P. (1971): Nature and inheritance of self-incompatibility in *Capsicum pubescens* and *Capsicum cardenasii*. *Hilgardia,* **40**, 459–470.

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Review

SELECTION OF PAPRIKA IN ANCIENT TIMES AND TODAY

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Some idea of what paprika first looked like can be obtained from a relief found on the Tello Obelisk, thought to have been carved around 800–1000 AD. The introduction of paprika into Europe can be dated from the first voyage of Christopher Columbus to America in 1492. Portuguese ships carried paprika from Spain to Arabia, and from there it spread to all the areas conquered by the Ottoman Turks, including Hungary (Andrews, 1984). The first large-fruited (Kalinkói, Várnai), tomato-shaped and horn-shaped types were introduced into Hungary by Bulgarian market-gardeners in the late 19th century.

These market-gardeners maintained their paprika varieties through the positive selection of individual plants. The first organised breeding of vegetable peppers is linked with the name of Lambert Angeli (1916–1971), while the most successful paprika breeder in Hungary was István Túri (1933–1999).

No ready-made paprika lines are available on the market, so paprika breeders use a greater proportion of lines of their own breeding than those working with other species. It is an advantage, however, that in paprika many quality traits can be selected on the basis of phenotypical traits, so in many cases visual evaluation can be employed in place of the far more costly measurement of performance traits. A further advantage is that the paprika species behaves decisively as a self-fertilised crop, the plants require little space, and the species is cosmopolitan. To improve selection efficiency, an environment is required that accentuates differences for the traits to be selected. In Hungary this can best be achieved in a field environment, which is also less costly.

The following traits can be tested in field nurseries: tolerance (CMV, *Xanthomonas*, etc.), horizontal resistance traits (purple nodes, leathery leaves, etc.), development rate, abiotic stress tolerance, susceptibility to sunburn and purple fruit, undesirable fruit shape and surface, lack of pungency (C gene) and undesirable flavour traits.

The following traits can be selected either in the field or in the greenhouse: yield potential (fruit size, number of fruit/plant, flesh thickness), plant height, regeneration ability, Susceptibility to Ca spots, white colour, basic colour of biologically mature fruit, determinate growth.

Traits that can only be selected under controlled conditions: sensitivity to light deficiency, vertical resistance genes.

An important practical rule for selection is that more costly techniques should only be applied after the number of plants has been reduced using cheaper selection methods.

Key words: visual selection, self-fertilisation, artificial selection environment, field selection

History of paprika breeding

Archaeological digs in Central and South America, in Tamaulipas and Tehuacan, revealed paprika remains dating back to 7000 BC (MacNeish, 1964), while data on the domestication of the species are available from 2500 BC (Bird, 1948). Some idea of what paprika first looked like can be obtained from a relief found on the Tello Obelisk, thought to have been carved around 800–1000 AD, during the Chavin culture in Peru (Tello, 1960).

The introduction of paprika into Europe can be dated from the first voyage of Christopher Columbus to America in 1492. In his first letter to the Spanish monarchs, Ferdinand and Isabella, written in 1493, Columbus mentioned a very hot spice that the Indians ate with meat. A detailed description of the paprika plant was given by the doctor, Dr Diego Alvarez Chanca, who accompanied Columbus on his second voyage. Portuguese ships carried paprika from Spain to Arabia, and from there it spread to all the areas conquered by the Ottoman Turks, including Hungary (Andrews, 1984).

In Hungary, vegetable paprika types were unknown until the late 19th century. The first large-fruited (Kalinkói, Várnai), tomato-shaped and horn-shaped types were introduced into Hungary in the late 19th century by Bulgarian market-gardeners, who first settled in the region of Szentes and later spread throughout the country, taking with them not only the technology required for growing paprika, but also various types of varieties. These market-gardeners maintained their paprika varieties through the positive selection of individual plants (Czibulya, 1987).

Until the late 1940s, no organised breeding was carried out in Hungary on vegetable peppers, but selection had already led to the evolution local varieties in various parts of the country. The Kalinkói type was a light green, thin-fleshed, sweet paprika, which would now be referred to as blocky. The original type is no longer cultivated in Hungary, but it was probably related to the Kalinkov varieties still grown in Bulgaria. The sweet variety Elephant Trunk, selected from a horn-shaped paprika, Sipkai Hot Green grown in Orosháza, in S.E. Hungary and still included in the Hungarian variety list, is no doubt an original type. Another type with a predominantly original genome, probably arising from the Bulgarian population Sipkai White Pungent, is the Improved Bogyiszlói variety, a hot, white, thick-fleshed paprika, the ancestor of which was maintained by a form of hybridisation, whereby plants with apple-shaped and elongated fruit were planted in alternate rows, and the fruit of the various types were marketed separately. Seed from the mixed pollinated stands were mostly taken from plants which exhibited the Bogyiszlói traits.

The greatest service to Hungarian paprika breeding was done by gardeners in the village of Cece, who, by the 1920s, had selected a type of paprika with broad shoulders, an elongated conical shape and an extremely rare white colour, which probably arose from progeny populations of peppers from Varna and

Plovdiv. This type is still known as the Cecei type (Fig. 1). This type formed the basis of the most widespread vegetable paprikas grown in Hungary today, and its excellent flavour led to paprika being an essential part of the Hungarian cuisine. The first Cecei paprikas were pungent, but were later selected in various directions.

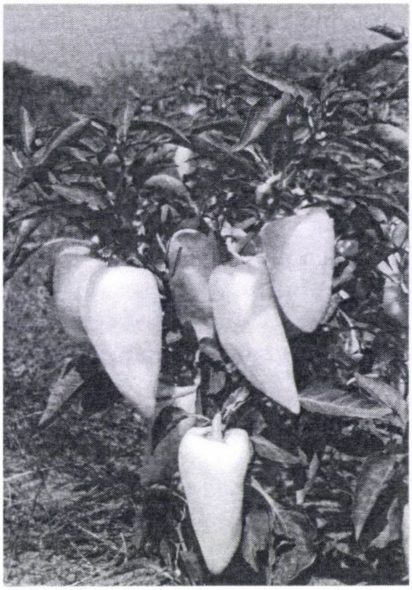

Fig. 1. The "Cecei" type of paprika

The first organised breeding of vegetable peppers in Hungary is linked with the name of Lambert Angeli (1916–1971), who started selecting populations of the Cecei type in the Horticultural Research Institute, the predecessor of today's Vegetable Crops Research Institute, in 1949, registering the first sweet, white, large-fruited variety under the name of Cecei Sweet 3 in 1953. At that time the average weight of Grade I fruit was 50 g (Angeli, 1959). With the cultivation of this variety, national yield averages were doubled and there was an enormous increase in the consumption of Hungarian paprika, which almost reached the current level by the 1960s (200,000 t/year). The original Cecei genome is preserved as a gene bank accession in the Improved Cecei variety. Angeli also initiated the breeding of varieties and lines with determinate growth using *C. annuum* cv. *fasciculatum*. After his death in 1971 this work was continued by his colleagues, culminating in the nation-wide production of the Fehérözön variety in the 1980s. As the result of heterosis studies begun in the 1950s (Angeli, 1957; 1959), the first Hungarian paprika hybrid varieties were developed in the early 1960s: the hot pointed variety H-2 and the sweet white variety H-1. The male line of these hybrids, which has extremely rapid development, is still used in breeding.

The other major name in Hungarian paprika breeding was István Túri (1933–1999), who worked first for the University of Horticulture and later for the Produkt Co. Ltd. In the 1970s the yields of varieties of the Cecei type declined due to increasing CMV infection, while the cultivation of paprika under polythene underwent rapid development. The first major variety bred by Túri, Soroksári Hajtató, soon became dominant in greenhouse production, where a number of new technological innovations were made. In the 1980s this variety was replaced both in the greenhouse and in the field by Fehérözön, which contained the *L1* gene for tolerance of spider mites and had determinate growth. Túri's first hybrid variety, HRF (developed using a female line selected from Soroksári Hajtató and containing the *ru* rugose leaf gene, and Fehérözön as male line) became the market leader in the 1990s. All the numerous hybrids later produced by Túri and his colleagues involved rugose-leaved female lines with outstanding general combining ability.

Selection of vegetable peppers today

Not only are no ready-made paprika lines available on the market, but no initial stock has a phenotype anything like that of the Hungarian variety types. For this reason emphasis is placed on faster, more rational breeding techniques based principally on material developed by individual breeders.

Paprika, however, has certain advantages that can be exploited by breeders. In this species many quality traits can be selected on the basis of phenotypical traits, so in many cases visual evaluation can be employed in place of the far more costly measurement of performance traits. A further advantage is that the paprika species behaves as a self-fertilised crop, with only a low percentage of open pollination. The plants require little space (in extreme cases they can produce fruit and ripen seeds at a density of 20–30 plants per m^2) and can be sown at any time of the year.

The question arises of where breeding stock can be selected more efficiently. One school of thought states that selection should be made in the environment in which the plants will be grown in order to accumulate factors promoting adaptation to the given environment. In this case varieties for greenhouse cultivation should be selected in the greenhouse and those intended for field use in the field. The majority of breeders, however, consider that neither plants grown under luxury conditions nor those grown under poor conditions are suitable for selection as in both cases the deviation for the traits to be selected is limited, making it difficult to find genotypes with above-average values. If varieties intended for greenhouse production are selected under polythene, many of the fruit deformities caused by cultivation under extreme conditions will not be exhibited.

In some cases the targeted growing environment is so specific, or differs to such an extent from the breeding environment, that selection can only be effective in the growing environment. In general, however, paprika is a cosmopolitan species and can be selected for most economically important traits under a wide range of environmental conditions. Varieties tolerant of low light intensity and low temperature, intended for cultivation under polythene in the Mediterranean winter, are selected by Dutch companies in breeding stations set up in the Mediterranean region, but vegetable pepper hybrids bred in Western European greenhouses can be successfully grown in the field under Hungarian conditions.

In many parts of Europe, including Hungary, a large proportion of the vegetable peppers are grown under polythene. The variety traits required by the two methods of production are identical, with the exception of sensitivity to light deficiency. This is confirmed by the vast number of varieties that can be grown equally successfully in the greenhouse and in the field. It is thus obvious that the best place for selection does not depend on where the variety will be cultivated.

The most important criterion for effective selection is the provision of a homogeneous environment where the desired traits will be manifested over a wide range, thus allowing genotypes with the largest positive deviation for a given trait to be selected. Under Hungarian conditions the major traits of paprika can best be selected under the following conditions:

– Lines can best be distinguished for their sensitivity to light deficiency on the basis of their vegetation period when sown in late September (Zatykó and Ács, 1980).

– Selection for vertical resistance genes (*L3, L4, Tsw, Bs2*, etc.) can be carried out on the basis of hypersensitive symptoms after artificial inoculation under controlled climatic conditions, but not after natural infection in the field (Zatykó, 1996).

– Tolerance (to CMV, *Xanthomonas*, etc.) can only be tested in the field in years with severe (and homogeneous) natural infection.

– Horizontal resistance traits (purple nodes, leathery leaves, etc.) are best manifested in the field.

– Development rate (vegetation period under optimum light conditions, earliness) can be selected after sowing in the field in April or May, or in a controlled environment, on the basis of the appearance of the first branching (the potential location of the first flowers). The development rate can also be judged later from the date of flowering and seed setting at the first branching point, or from the state of development of the fruit, i.e. at any stage from the appearance of the first branching until picking. If for any reason the fruit is aborted from the first branching point, it is difficult to judge the rate of development. Regardless of whether fruit is set or not, the appearance of the first branching is always proportional to the development rate at any sowing date. Considering the very close negative correlation between earliness and yield potential (fruit size), selection should not be made purely on the basis of earliness before the fruit parameters and number of fruit per plant are known.

Rough, phenotypical selection for the main components of yield potential: fruit size, number of fruit per plant and flesh thickness, can be carried out both in the field and in the greenhouse in average stands.

Thick flesh is found under smooth skin and thin flesh under ribbed skin, and the flesh thickness can be judged by touching.

Plant height can easily be measured in fully developed stands in the field or greenhouse. The internode length on which plant height depends is an important trait, but may exhibit considerable deviation even within a single plant, for various reasons, especially in plants grown under polythene. The mean length of the internodes is proportional to the plant height. The serial number of the node where the first branching takes place is genetically determined between fairly narrow limits.

The regeneration ability of the paprika plant, which determines the rate at which development is renewed after stress, is an important aspect of yield potential. The importance of regeneration ability was recognised in the 1970s when bunched varieties appeared, since the first varieties with determinate growth had poor regeneration ability, i.e. the first bunch of fruit overtaxed the plants to such an extent that they stopped growing, the tissues aged, and even after the fruit were picked the plants were incapable of regrowth. The variety Fehérözön made it possible to describe the trait of regeneration ability, as in this variety determinate growth was associated with continuous growth, seed setting ability and rapid regeneration after stress (Zatykó and Moór, 1982). Regeneration ability means that the aging plant retains the juvenile state to a certain extent, and this is generally accompanied by tolerance of mites (*Tetranichus urticae*) (Zatykó and Martinovich, 1986). Good regeneration ability allows the plant to overcome technological or environmental stress effects, and selection for this trait is best based on the appearance, number and quality of new seed setting on unharvested plants bearing a large number of fruit, under field or greenhouse conditions (or possibly by artificially infecting plants with mites in the 4–5-leaf stage).

Yield potential is greatly influenced by the behaviour of the plants under extreme conditions, i.e. by their abiotic stress tolerance. Under Hungarian conditions, both in the field and under polythene, paprika plants are obliged to spend most of their lives under suboptimum conditions (low temperature in spring, autumn and at night, summer heatwaves, intense insolation, high salt concentrations, temporary water deficiency, etc.). Despite the very complex nature of stress tolerance, it can be evaluated reasonably well on the basis of certain traits. The response to extreme conditions can best be studied on field plants, the exposure of which is greater than that of greenhouse plants, especially in early autumn. The behaviour of lines or combinations to stress factors can be evaluated from the quantity and quality of the fruit on the side branches of older plants, from the type of fruit produced in autumn, from the size, form and colour of fruit at the branch tips in autumn, and from that of fruit set during heatwaves or in cold weather.

Susceptibility to sunburn can be adjudicated in the field, while water/nutrient uptake anomalies and the susceptibility to Ca spots during atmospheric drought can be evaluated in either the field or greenhouse.

Selection for white colour is most efficient when the plants are forced very early or in late autumn, or on plants grown in the field, especially in autumn. Under these conditions the otherwise white (yellowish white) fruit will turn green if they are liable to do so. Some combinations of the *sw* genes responsible for white colour (Csilléry, in Somos, 1985) include shades of green, and experience shows that variants tending to turn green generally have larger fruit, so it is advisable to select for larger fruit size in an environment which will reveal the tendency to turn green.

Several genes are responsible for the development of purple anthocyanin coloration in the fruit (*A, Mo, R1*, etc.). However, the presence of some of these genes is only manifested under extreme environmental conditions. The tendency of the fruit to turn purple is most clearly revealed on the sunlit side of peppers grown in the field in autumn and is probably manifested due to the great heat fluctuations on the fruit surface. The apple- and tomato-shaped forms tend to turn purple at the tip, but this is always caused by environmental effects stimulating vegetative growth, and never on the sunlit side. The higher anthocyanin content in other plant organs is not manifested in the fruit, so greater purple coloration of the nodes and hypocotyl (which may be a favourable trait) is not correlated with fruit colour. Purple fruit colour, which results in plants being rejected, is not observed in the *al/al* genotype.

The basic colour of biologically mature fruit (red, salmon, pink, orange, yellow, cream) can be efficiently selected in any environmental background, due to the low number of genes responsible for this colour (y, c_1, c_2) (Lippert et al., 1965). The intensity of the colour is determined by the quantity of pigment components (carotenoids), a polygenic trait. Selection for genotypes inheriting high pigment content can best be carried out under polythene, which has an unfavourable effect on pigment content, and on chemical quality in general.

Defects in fruit shape and surface (deep-set stalk, unsatisfactory shoulder shape, ribbed surface, flat, curved or indented fruit, unsatisfactory fruit index, too thin or too thick skin, thin flesh, unsatisfactory flesh/stalk ratio, pointed types with an introverted end, fruit with a beak-shaped protuberance or blunt end, thickening of the style, blocky types with a pointed end, ribbed surface, dull or cracked skin, difficult to pick, etc.) are the most frequent reasons for the rejection of plants, lines or combinations, partly because many such defects arise and partly because consumers tend to prefer certain traditional forms. Quality criteria are becoming constantly stricter, and some traits associated with certain variety types are now classified as defects (ribbed surface, thin epidermis, broad shoulder, very large or very small fruit, etc.). The best background for selection against shape and surface defects is field growing, where these defects are accentuated, sometimes amounting to deformities, particularly if the stand is in below-average condition. The manifestation of these traits is generally extremely environment-dependent. Some are completely masked when the plants are in good condition and may not be manifested until the variety is entered in state trials. Many defects in fruit shape (curved or indented fruit, lack of shoulders, asymmetric shape, beak-like protruberance, fleshy style) are the consequence of deficient fertilisation, asymmetric ovary location, or partial or complete parthenocarpy, while other genotypes have normally shaped fruit even in the case of poor seed setting or parthenocarpy. The best selection criterion for types with good fertilisation and good fruit shape is generally the seed setting in the field in autumn and the shape of the fruit on the branch tips. Although the appearance of shape and surface defects is very environment-dependent, the tendency to such defects is strongly inherited. Combinations with this tendency should thus be rejected in the early stages of breeding, if possible in the F_1 of line-improving or line-developing crosses. Shape and surface defects may also be manifested if the parental lines were free of such defects, as many are the consequence of partial genetic incompatibility between the parents. Another clearly perceptible manifestation of this incompatibility is the appearance of "blind" shoots that do not form a bud in F_1 seedlings and in many further generations. Unless they are particularly valuable for some other reason (e.g. interspecific hybrids), such combinations should be rejected. The majority of shape and surface defects can only be moderated by selection, but not eliminated. When lines containing shape defects involving shoulder shape, ribbed surface, beaks or tip shape are crossed with perfect forms, the defects are frequently manifested in the progeny populations, so combinations with shape defects can in many cases be eliminated in the F_1.

The great environment dependence of the shape of the tip (pointed or introverted) can only be overcome by selection under extremely stressful conditions. The *O* gene responsible for introverted fruit tips is dominant below a certain fruit index (length/width), but the fruit may well become pointed as the result of environmental effects. Apple-shaped paprika tends to be conical when fruit are set in autumn, while blocky types that are not sufficiently stable may be pointed under stress conditions (in autumn in the field, or when forced early). The opposite may be observed for the pointed Cecei types if the genotype is not sufficiently stable. The introverted tip is more stable in four-veined than in three-veined types, for those with a smaller fruit index and for thick-fleshed blocky fruit, while the pointed tip is more stable in two-veined than in three-veined fruit and for more elongated types.

Determinate growth (the shoots form nodes that stop growing and develop two or more flowers after branching for a certain number of times, determined by the environmental conditions) has been considered since the work of Deshpande (1944) to be a monogenic recessive trait (*fa*), but it is manifested in many types ranging from super-determinate (stopping growth at the first branching, where a bunch of flowers is formed) to types which grow continually, forming many branches before vegetative growth ceases. Vegetable peppers with determinate growth (Csokros felálló, Csokros csüngő) were first developed in Hungary by Angeli (1968) using the wild type *C. annuum* cv. *fasciculatum*. These super-determinate types could be cultivated as short-period crops. The super-determinate nature of the spice paprika variety Kalocsai D 601 allowed the crop to be harvested in a single operation. The variety Fehérözön (a variety used as a UPOV example under the name Feher), suited to the new forcing technology and long-term production introduced in the 1980s, was also of the bunched type, due to its tendency to continually form new side-branches. Types with determinate growth are now used mainly as parental lines for hybrid varieties, as the *fa* gene enhances the generative nature of the hybrid and the frequency of nodes setting two fruits even in the heterozygous state and also prevents the unauthorised multiplication of the F_1. The selection of homozygous plants with determinate growth from the segregation population is simple both in the field and under polythene, but at later stages the determinate growth type only becomes obvious in the greenhouse, generally in the vegetative stand, after the plants have produced a large number of branches. The fixation of a genotype carrying genes for a certain level of determinate growth needs to be carried out in a generative stand, where undesirable super-determinate forms can be rejected.

The *up* gene is responsible for the erect or drooping nature of the fruit, but large-fruited varieties with the erect *up/up* genotype can only be identified reliably from the growth habit of the flowers. Selection may be disturbed by the fact that the up/up+ genotype, which normally droops, may become semi-erect or transitional in some combinations in response to environmental effects (Csilléry in Somos, 1985). Nor is there a satisfactory explanation for the observation that if the erect parent in a drooping × erect combination is a DH line, all the heterozygous F_1 plants will exhibit the erect phenotype. The erect growth habit is associated with more regular fruit shape, but it has the disadvantage that the fruit surface is more exposed and the fruit are more difficult to pick. Although the Cecei variety models usually give preference to droopy growth habit and the majority of initial combinations are droopy, due to the dominant nature of this trait, the most successful varieties of this type in recent decades (Soroksári Hajtató, Fehérözön, HRF F_1, Ciklon F_1) were all erect, suggesting that other positive factors (influencing yield potential, short vegetation period) may be linked to the erect growth habit.

Increasing interest is being expressed in the selection of paprika on the basis of seedling traits, using genetic markers, as this would allow a larger number of plants to be tested and would lead to a saving on plant growth costs. Codominant markers can be used to select plants carrying resistance genes in the homozygous state, allowing a year to be saved in the case of monogenic dominant resistance genes. For recessive monogenic resistance, backcrosses can be carried out in the BC F_1 on heterozygous plants selected using markers. Very few factors are known to be linked to cotyledon traits such as hypocotyl anthocyanin discoloration (proportional with horizontal resistance), leaf colour (which reflects the fruit colour), leaf width (proportional to fruit width) or erect (Fehérözön) habit. Experiments carried out by Mulge and Anand (1997) showed that conclusions on the general combining ability of lines and the specific combining ability of combinations for yield components could be drawn from the seedling growth rate and the height of young plants. Selection for the *al* gene can be made in the cotyledon stage, test combinations leading to blind shoots can be eliminated, and selection can be made after artificial virus inoculation.

The pungency of paprika is a monogenic dominant trait (*C*). The hotness of the fruit is determined over a very wide range by the quantity of capsaicinoids (Deshpande, 19350). Daga and Tamura (in Zatykó, 1979) found that capsaicin was also present in sweet varieties, but at a level of around 150 mg, compared with around 1000 mg for pungent varieties. The capsaicin content of the fruit depends on environmental factors to such an extent that varieties producing sweet fruit when grown under polythene in winter in the Mediterranean have pungent fruit when grown in the field in summer. Selection for pungency can best be carried out by early forcing, where variations in pungency from extremely mild to very hot can be readily distinguished. For the same reason, however, it is risky selecting for sweet fruit under polythene, as the pungency of plants having a low capsaicin content in a vegetative environment, but carrying the *C* allele, may remain masked. Sweet types should be selected under field conditions.

Some of the most valuable assets of Hungarian paprika varieties are their aromatic compounds, yet breeders have little knowledge on how these can be preserved. It is quite clear that white varieties that contain much of the original genome have special flavour and aromatic compounds that the majority of consumers prefer to those present in dark green peppers. Some 80% of blindfolded tasters could distinguish the colour on the basis of the taste and chromatographic analyses also demonstrated compounds present only in varieties related to Cecei. It is also clear that the pleasant flavour of white Hungarian peppers is not linked closely to the white colour, since white varieties of non-Hungarian origin were given a poor evaluation in organoleptic tests. The aromatic compound found by Buttery et al. (1969) to be characteristic of paprika, which has since been produced synthetically (2-methoxy-3-isobutylpyrazine) was extracted from the dark green variety Californian Wonder, so this is not the substance that must be sought by Hungarian breeders. Flavour and aroma can only be selected by organoleptic tests, which are basically restricted to the initial materials and to new varieties or lines that have already been screened for other traits. Flavour defects are manifested to the greatest extent in field cultivation in autumn.

The thickness of the fruit epidermis is determined by the number of parenchyma cell rows surrounded by the chitin layer developing in the intercellular spaces. In thin-skinned varieties there are 1–2 rows and in thick-skinned varieties 4–5 (Fischer, 1974; Zatykó and Fischer, 1992). Epidermis thickness is a polygenic trait with little environment dependence. From the point of view of consumer tastes and healthy nutrition, an easily digestible skin thinner than 40 microns is preferable (Fehérözön), but such varieties have a shorter shelf life, so traders prefer varieties with thicker skins. The exact skin thickness is measured after cooking, when it can be easily separated from the flesh, and is classified as thin (< 40 μ), medium (40–80 μ) or thick (> 80 μ). The latter category is unpleasantly thick for fresh consumption.

An important practical rule for selection is that more costly techniques should only be applied after the number of plants has been reduced using cheaper selection methods, especially visual selection. The best lines or combinations can then be included in replicated performance trials and submitted to organoleptic analysis, before being tested in large-scale cultivation.

References

Andrews, J. (1984): *Peppers. The Domesticated Capsicums*. University of Texas Press, Austin.

Angeli, L. (1957): Heterózis paprikatermesztési kisérletek. (Experiments on heterosis for paprika production.) *Kert. Kutató Intézet Évkönyv*, **2**, 131–140.

Angeli, L. (1959): *Paprikatermesztés*. (Paprika production.)Mezőgazdasági Kiadó, Budapest.

Angeli, L. (1968): *Paprikatermesztés*. (Paprika production.) Mezőgazdasági Kiadó, Budapest.

Buttery, R. G., Seifert, R. M., Lundin, R. E., Guadagui, D. G., Ling, L. (1969): Characterization of an important aroma component of bell peppers. *Chem. Ind. London No. 15.*

Columbus, C. (1493): The letter of Columbus to Luis de Sant Angel announcing his discovery (1493). *Harvard Classics*, **43**, 22-28, 1910.

Czibulya, F. (1987): *Bolgárkertészet magyar földön*. (Market gardening in Hungary.)Mezőgazdasági Kiadó. Budapest.

Deshpande, R. B. (1935): Studies in Indian chillies. 5. Inheritance of pungency in *Capsicum annuum* L. *Ind. Jour. Agr. Sci.*, **5**, 513–516.

Deshpande, R. B. (1944): Inheritance of bunchy in chili (*Capsicum annuum* L.). *Ind. J. Gen. Pl. Breed.*, **4**, 54.

Fischer, I. (1974): The exocarp and fruit quality in pepper varieties. In: *Eucarpia. Genetics and Breeding of Capsicum*. Budapest, pp. 51–58.

Lippert, L. F., Bergh, B. O., Smith, P. G. (1965): Gene list for pepper. *The Journal of Heredity*, **56**, 30–34.

Mac Neish, R. S. (1964): Ancient Mesoamerican civilization. *Science*, **143**, 531–537.

Mulge, R., Anand, N. (1997): Prediction of heterosis and combining ability for yield and yield characters at seedling stage in sweet pepper (*Capsicum annuum* L.). *Ind. Jour. of Genetics and Plant Breeding*, **57**, 180–185.

Somos, A. (1985): A paprika, *Capsicum annuum* L. (Paprika, *Capsicum annuum* L.) *Magyarország Kultúrflórája 54.* Akadémiai Kiadó. Budapest.

Tello, J. C. (1960): *Chavin: Cultura matriz de la civilizacion andina*. (Chavin: Basic culture of the Andean civilisation.) Univ. San Marcos Press, Lima, Peru.

Zatykó, L. (1979): *Paprikatermesztés*. (Pepper Production.). Mezőgazdasági Kiadó, Budapest.

Zatykó, L., Ács, I. (1980): Research and application of two factors determining the breeding season of pepper. *IV. Meeting of the Capcicum Working Group of Eucarpia*, Wageningen.

Zatykó, L., Moór, A. (1982): Fehérözön – Új paprikatípus, új lehetőségek a nemesítésben. (Fehérözön – New paprika type, new possibilities for breeding.) *Kertgazdaság*, **14**(2), 61–69.

Zatykó, L., Martinovich, V. (1986): Resistance to the red spider mite (*Tetranichus urticae* Koch.) in the "Fehérözön Synthetic" pepper variety. *VII. Eucarpia Meeting Capsicum and Eggplant*, Saragosa.

Zatykó, L., Sasvári, M. (1992): Moderation of the negative correlation between size and growth rate in new pepper varieties for comsumption. *Capsicum Newsletter (Special Issue)* VIII. Eucarpia Meeting, Genetics and Breeding on Capsicum and Eggplant, Rome. pp. 100–105.

Zatykó, L., Fischer, I. (1992): A terméshéj vastagságának szerepe az étkezési paprika termesztésében, fogyasztásában és a nemesítésben. "Lippai János" tud. ülésszak előadásai. (Role of skin thickness in the production, consumption and breeding of vegetable paprika.) Kert. és Élelmiszeripari Egyetem Kiadványai, Budapest. pp. 912–915.

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Review

PEPPER (*Capsicum annuum* L.) BREEDING METHODS AT THE TURN OF THE CENTURY

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One important aim of hybrid breeding is to exploit the heterosis effect appearing in the F_1. Nevertheless, the breeders of commercial F_1 hybrids have no real information on the extent of heterosis manifested in the combinations they produce, since the mean value of the combination in question is never compared with that of the parents or of the better parent, but only with that of the most popular control variety it is hoped to surpass. The complex variety value of a new hybrid should be greater than that of the control. In the case of pepper hybrids the factors that make up the complex variety value can be divided into four groups: the early and total yield potential predicted from the individual value of the parents (P), special consumption and production traits resulting in F_1 quality (Q), F_1 resistance value (R) and the heterosis effect (H). The importance of these four factors in the complex variety value of a given pepper hybrid may be summed in innumerable variations, but the individual yield potential and quality traits of the parents are of outstanding importance. This is the basis, without which combining ability, resistance value and heterosis effect will remain unexploited.

When selecting pepper lines for combining ability, risks may be involved in over-strict selection for general combining ability alone, so a combined crossing system involving a carefully constructed partial diallel is normally employed to obtain information on the general combining ability of lines preliminarily screened for individual plant performance and on the specific combining ability of their combinations. Cross-breeding aimed at the development of parental lines and constant varieties makes use of single crosses, crossing series, backcrossing and resistance breeding.

Key words: quality traits, heterosis, line development, general and specific combining ability, cross-breeding, resistance

Breeding of F_1 hybrid varieties

Investigations conducted by many authors prove unanimously that a heterosis effect can be achieved in peppers for the characters plant height, seed quantity per fruit, number of fruit per plant, fruit size, and the combined effect of these on the early and total fruit yield per plant (Daskalov, in Banga and Banga, 1998; Ahmed and Muzaraf Hurra, 2000). However, from the point of view of heterosis in the yield the most important factor is the number of fruit per plant, while the other yield components do not play a major role (Kaul and Sharma, 1988). Heterosis levels between 15 and 50% in the early and total yield have been reported by various authors. The yield components are controlled by additive and non-additive (dominance, overdominance, epistasis) gene effects, the influence of which varies considerably as a function of the trait, environmental factors and the genotype of the line or variety.

As in other crops, one major argument in favour of hybrid breeding is that the development of F_1 hybrids is the simplest way of combining various types of dominantly inherited resistance and other traits.

In self-fertilised species such as pepper some of the positive traits exhibited in the F_1, particularly those controlled by additive gene effects, can be fixed in the progeny lines (Kuckuck et al., 1985; Doshi and Shukla, 2000a). For instance, despite the fact that the mean fruit mass of white pepper lines exhibits a close negative correlation with the development rate, it has almost doubled over the last 50 years, while the development rate has remained unchanged (Fig. 1; Zatykó and Sasvári, 1992).

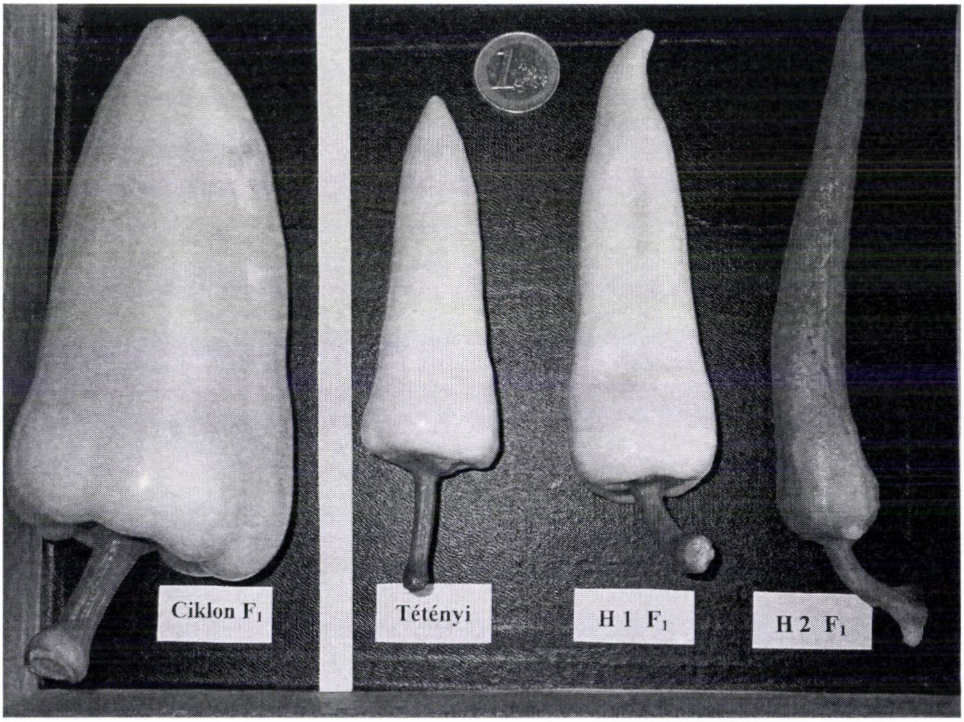

Fig. 1. Fruit of Hungarian pepper varieties

In the case of pepper this ensures the continuing success of line improvement, but, thanks to the substantial number of additive components in yield traits, there would also be considerable scope in the breeding of constant varieties. The fact that breeding companies prefer to market F_1 varieties can be attributed mainly to the question of variety rights. This is confirmed by the results of experiments on the inheritability of morphological and phenological traits in peppers, indicating that the mean fruit mass (and its components), the mean fruit number per plant and the resultant yield, the time to flowering and the mean plant height all exhibited high heritability indexes (h^2), allowing breeders to achieve good selection gains. The moderate to low h^2 values found for fruit length and diameter and for the total soluble dry matter content will lead to slower selection gain.

General scheme of hybrid breeding

Breeders dealing with open-pollinated species cultivated on large areas (maize, sunflower, etc.) work with thousands of lines, which may be commercially available lines with known genetic value, lines bred by the breeder, or new homozygous lines created by repeated self-pollination from initial populations developed by crossing elite lines with known genetic value. Test combinations are made with these lines using one or two known testers and the lines with the best general combining ability are selected based on the results of replicated performance trials on the test combinations. Those with the best specific combining ability are then chosen from replicated performance trials on F_1 combinations from partial diallel crosses on the lines with the best general combining ability. Unlike self-pollinated species, the majority of open-pollinated species exhibit inbreeding depression after 3–5 generations of self-pollination, partly because recessive genes with a lethal effect or coding for reduced vitality are present in the homozygous state. The lines thus have poorer performance, making it impossible to determine their individual value. Inbreeding depression thus means that the number of lines cannot be reduced by line selection on the basis of individual value prior to crossing with testers. Instead, the number of lines is reduced by the early testing of general combining ability (primarily for yield potential), usually in the first or second generation of inbreeding.

Value components of pepper hybrid varieties

An important, if not the most important, aim of hybrid breeding is to exploit the heterosis effect appearing in the F_1. Nevertheless, the breeders of commercial F_1 hybrids have no real information on the extent of heterosis manifested in the combinations they produce, since the mean value of the combination in question is never compared with that of the parents or of the better parent, but only with that of the most popular control variety it is hoped to surpass. The aim of the breeding company is to develop a new hybrid with a complex variety value greater than that of the control. In the case of pepper hybrids the factors that make up the complex variety value can be divided into four groups (Fig. 2):

The early and total yield potential predicted from the individual value of the parents (*P*)

Special consumption and production traits resulting in F_1 quality (*Q*)

F_1 resistance value (R)

Heterosis effect (*H*)

The majority of the components of parental yield potential, such as number of fruit per plant, mean fruit mass, tendency to set two fruits at each node, quantity and quality of yield under extreme conditions (in autumn, in the field, on side shoots, etc.), regeneration ability, sensitivity to light deficiency, etc., need to be present on average in the parental lines at the level required for the variety model, i.e. at a value above that of the control. A smaller or larger extent of heterosis can only be expected with certainty for a few of these traits, such as the number of fruit per plant and, to a lesser extent, the mean fruit mass. After visual evaluation and performance trials in hybrid combinations, the relatively small number of lines selected for outstanding individual performance and good general combining ability should then be tested in performance trials to obtain comparative data on their early and total yield ability, which can be used in planning hybrid combinations.

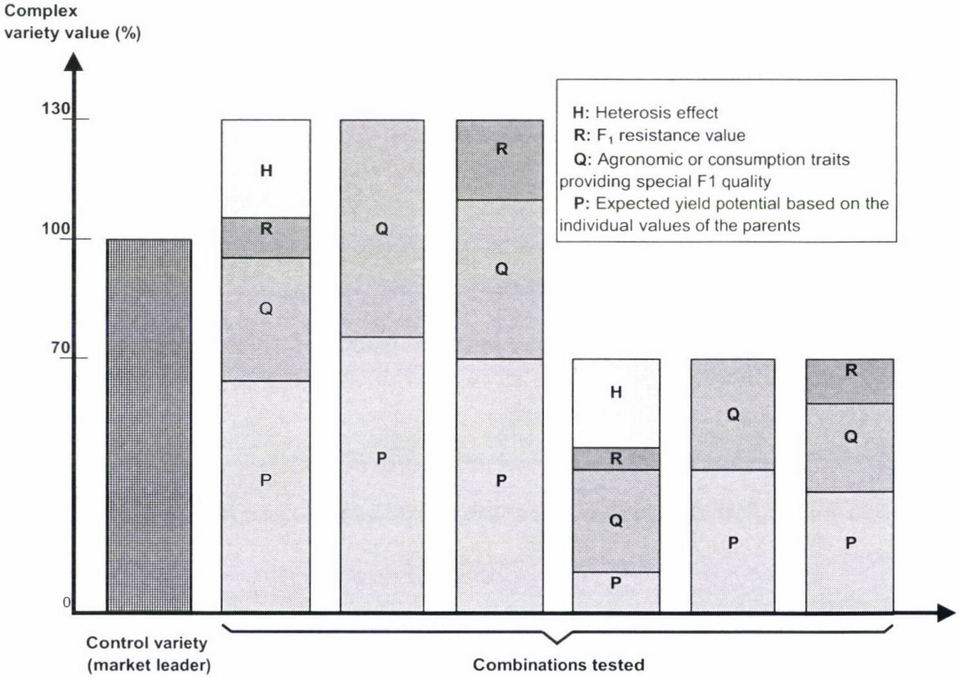

Fig. 2. Factors determining the value of F_1 combinations

The special consumption and production traits resulting in F_1 quality include completely new traits as well as those already known to have a positive influence on quality, leading to above-average values. New traits may be extra shiny skin, extremely large fruit, very regular shape, thin skin, very thick pericarp, unusual flavour, or a new form or colour specific to a new product. The majority of special traits influencing F_1 quality are negative traits that, if present to a certain extent, exclude the combination from further tests. These include uneven surface, irregular shape, dull skin, colour deviations, indented fruit, fruit with a beak-shaped protuberance, blocky types with a pointed end, pointed types with an introverted end, fruit with anthocyanine coloration, sloping shoulders, deep-set calyx, unsatisfactory fruit index, tendency to fruit shortening, Ca spots or sunburn, black blossom end, petals remaining on fruit, undersized fruit, large deviations in fruit size, thin flesh, thick skin, poor fruit consistency, unpleasant tang, excessive side-shoot formation, difficult to pick, etc. The large number of compulsory quality traits (some formal, some substantial) and of unacceptable traits involved in pepper breeding, and the frequency with which the latter occur is one reason why visual evaluation represents the first screening of lines and combinations. A large proportion of the lines and combinations tested (in some cases 70–80%) can be eliminated on the basis of quality traits (and the visual evaluation of the main components of yield potential) prior to the far more costly performance trials.

The F_1 resistance value is the product of various factors. These include the incorporation of monogenic vertical resistance (*L1, L3, L4, Bs* genes, *Tsw, N*), the *gds* gene coding for the general defence system, polygenic tolerance factors effective against various diseases (CMV, *Xanthomonas, Verticillium*), and horizontal resistance traits that improve general field resistance (dark green leaves, leathery epidermis, wavy leaf surface, deep purple nodes, strong branch system). The yield potential and quality of resistant varieties should not be poorer than those of non-resistant varieties, because in general growers are most concerned with yield potential when choosing varieties, and trust that the vegetation period will be free of infection. As farm sizes increase, environment-friendly technologies spread and greater emphasis is placed on yield reliability, commercial production will only be possible using pepper varieties with a high level of resistance.

Heterosis is most likely to be observed in F_1 pepper hybrids as an increase in the vegetative mass, or in its components (plant height, bushiness, number of branches) (Chang, 1977), or in the yield quantity (early and total), or in its components (particularly fruit number, but to a lesser extent fruit size and flesh thickness, which influence fruit weight) (Ahmed and Muzaraf, 2000). An increase in vegetative mass (plant height) is not the aim of most variety models, and may even be considered undesirable. However, it is positively correlated with the yield (Chang et al., 1977). During the selection of F_1 hybrids with the best special combining ability for yield, hybrid combinations manifesting a substantial heterosis effect for yield are obviously at an advantage, so it can be assumed that heterosis plays an important role in the complex variety value of the F_1 pepper varieties currently cultivated.

As seen in Figure 2, combinations may be selected in the course of testing even without a consideration of two of the four factors determining the complex variety value of pepper hybrids, the resistance value (R) and the heterosis effect (H), if the yield potential (P) and quality traits (Q) of the parental lines are of a sufficiently high standard and are associated with satisfactory combining ability. Conversely, if the yield potential of the parental lines is too low, their combinations will be rejected during testing even if there is a considerable heterosis effect. In the same way, if the parental lines have unsatisfactory quality traits, or if their quality traits are mostly polygenic, there is little chance that the positive traits of one parent will compensate for the negative traits of the other in combinations.

The importance of these four factors in the complex variety value of a given pepper hybrid may be summed in innumerable variations, but it can be seen from the above that the individual yield potential and quality traits of the parents are of outstanding importance. This is the basis, without which combining ability, resistance value and heterosis effect will remain unexploited.

Development of pepper lines

Valuable parental lines for the breeding of pepper hybrids can be obtained in a number of ways. As additive gene effects are dominant for the most important yield components, such as number of fruit per plant, earliness and total yield (Chang, 1977; Singh et al., 1982; Legesse, 2000), pepper breeders achieved considerable genetic gain in yield parameters (Fig. 1) within a relatively short period using conventional breeding methods (pedigree breeding), and in the fixing of these traits in constant varieties or lines. The existence of yield parameters determined by additive gene effects, with a high level of heritability, that can be selected easily due to close correlations, will facilitate the further efficient improvement of pepper breeding lines.

In addition to previously registered, elite lines, new lines can be started by crossing these and other lines (line development breeding) or from populations originating from existing F_1 varieties. For important variety types, a number of combinations (10–50, or even more) are set up simultaneously. Four important points should be considered when choosing parents for line development crosses. The lines derived from them should be suitable for the achievement of the variety model (including the possibility of complementation), they should have good general combining ability (being rich in additive gene effects), they should be genetically distant from each other, and it is an advantage if they are registered DH lines (which contain no lethal alleles). As early as the F_1 generation or in the F_2, where there are a large number of plants, information can be obtained from line development crosses on the quality and yield of the lines that can be produced from them, so breeding combinations leading to poorer progeny can be rejected at this stage. In some species the F_1 and F_2 generations of line development crosses have to be tested in replicated performance trials in order to determine which poorly performing combinations should be rejected, but in pepper this is unnecessary, as visual evaluation provides more adequate information on a number of traits, including yield potential, than measured data on a few traits. Later, too, both in the segregating generations and for homozygous lines, a substantial amount of visual selection can be carried out not only for traits that are undesirable for the given variety model, usually involving quality faults, but also for positive traits and quantitative values (yield potential), since inbreeding depression that masks traits indicative of individual values does not occur in pepper.

Another starting point for the development of parental lines for hybrids is the populations or lines arising from the last crosses in line improvement breeding (which may be multiple crosses or backcrosses). Lines developed as the result of strict backcrossing (using the same registered recurrent parent throughout) can be assumed to have the same combining ability as the recurrent parent.

The quickest way to develop homozygous lines is to use the doubled haploid (DH) technique, which can theoretically be applied in any progeny generation of the line development cross. The DH lines can be selected on the basis of phenotype prior to testing their combining ability.

Lines can also be obtained from constant varieties.

Selection of lines based on individual value

Although the real value of potential parental lines is represented by the extent to which their traits are manifested in their hybrids, they are strictly selected in the year(s) previous to their use in test crosses, partly to reduce the number of lines and partly to ensure that they do not contain traits undesirable for the variety model, while containing desirable traits to a high level. On the basis of data from the literature and his own experience, the breeder has a fairly realistic idea of the heterosis effect that can be expected for various traits in the F_1 variety. In large-fruited varieties, for instance, an early and total yield surplus of 15–25% can be expected compared with the better parent, and most of this will stem from a larger number of fruit rather than from an increase in the mean fruit mass. It is thus obvious that, even if the parental lines are expected to complement each other to a certain extent, the yield components (fruit size, flesh thickness, fruit number) of the lines tested should not be much below the model values required for the F_1 variety. In the case of additive traits it is even more important to reject lines with values below those required for the variety model, despite the possibility of the lines complementing each other. Line selection requires an accurate knowledge of negative traits with a high heritability index or with intermediate inheritance, or which may be enhanced by heterosis (plant height), and which are thus certain to be manifested in the hybrid and cannot be complemented (irregular, indented, greenish fruit, etc.).

From the point of view of heterosis, one of the most valuable aspects of the available pool of potential parental lines is the great genetic distance between the groups of lines (Allard, 1960). Phenotypic differences have no influence on the potential extent of heterosis (Sifriss and Sacks, 1980). Conclusions on the degree of relationship can be drawn from pedigrees reaching back into the distant past (and also using theoretical statistical methods), but information can be obtained most rapidly and reliably from line mapping using a large number of molecular markers.

Selection of pepper lines on the basis of general and specific combining ability

As in other crops, the search for lines with the best general combining ability and test combinations with the best special combining ability is an extremely important part of pepper hybrid breeding. However, the special features of the pepper plant and other important factors have led most breeding companies to depart from the "maize model" in the methods used to test combining ability.

In developing lines and evaluating combinations, selection based on visual observations is far more feasible in pepper than in the majority of crops. Within certain limits, the yield potential (early and total yield) can be judged from the number and size of the fruits, while quality traits, which are often a basic criterion, can often be evaluated more accurately by visual observations. Without the visual screening of the large number of lines and test combinations, pepper breeding companies would be obliged to significantly reduce the volume of breeding stock, as otherwise the measurement of 50–100 performance data (weekly or fortnightly picking, 4–5 commercial grades, fruit number, weight) in the pepper performance trials would take up a large proportion of the capacity. In the early stages of breeding, rapid visual evaluation is better than precise measurements (Allard, 1960), and this is also true of the first selection on test combinations.

The visual evaluation of pepper is promoted by the close correlation between yield potential and a number of traits with clear phenotypic manifestation, while the reliability of this classification is enhanced by the high heritability values of these traits.

During the first visual screening of lines and combinations for earliness (rapid development rate, low sensitivity to lack of light) the plants can be scored for branching, start of flowering, date of first fruit setting or start of biological maturity. However, when evaluating earliness it must not be forgotten that the general negative correlation observed in the plant kingdom between earliness and yield potential is also valid for pepper (Chang et al., 1977; Chung, 1981). The first visual screening for early and total yield quantity can be used to rank the lines and combinations on the basis of fruit number per plant, fruit size, and perhaps pericarp thickness and vegetative mass. Numerous studies have confirmed that yield potential can be judged from the fruit number, fruit size, vegetative mass and even from the length of the fruit shank, since all these traits are in positive correlation with the total yield (Chang et al., 1968; 1977; Chung, 1981), though the fruit number per plant is negatively correlated with the mean fruit mass and flesh thickness (Depestre et al., 1986). Several authors, including Doshi et al. (2001), have demonstrated that seedling traits are suitable for the estimation of combining ability for total yield.

The heritability values for earliness, fruit number per plant, fruit weight, total yield and plant height are all high (Chang et al., 1968a; 1977; Chang and Chung, 1979; Choi and Kim, 1986; Depestre, 1988; Depestre et al., 1989), so visual evaluation is not greatly affected by environmental effects.

According to Kuckuck et al. (1985) the separate testing of general combining ability can be omitted during the hybrid breeding of self-pollinated species, where the number of lines can be reduced; it is sufficient to carry out diallel crosses for the genotypes. It is also worth noting the findings of Kalloo (1988), who stated that the specific combining ability in crosses gives a better indication of the value of the parents than an examination of their general combining ability, while diallel crosses provide information on both general and specific combining ability. General combining ability is indicative of additive gene effects, while specific combining ability reveals the presence of dominance and epistasis (non-additive gene effects) (Kalloo, 1988). Pepper breeders are fundamentally interested in finding parental lines with good specific combining ability, i.e. with non-additive gene effects for as many major traits as possible. As general combining ability depends more on additive gene effects (Doshi and Shukla, 2000a), which in themselves will not make a combination better than the better parent and which are generally only valid for yield potential, strict selection based purely on general combining ability can be risky. The fact that a pepper line has only moderate general combining ability does not necessarily mean that it does not possess non-additive factors and other traits making it a good specific combiner.

According to Sprague (1946; in Allard, 1960) early testing is worthwhile if there are too many parental lines and if the emphasis is on yield potential, but not if visual evaluation is possible. Allard (1960) also cites Singleton and Nelson (1945), Richey (1945; 1947) and Payne and Hayes (1949), who reported that visual selection was effective in early testing, but warned that early testing might lead to the rejection of potentially valuable lines.

Kalloo (1988) raised the possibility of line selection prior to the evaluation of combining ability for vegetable crops, since a correlation was found between three data: the parental mean, the performance of the combinations, and the general and specific combining ability. This author also stated that selection based on hybrid performance was often just as successful as that based on specific combining ability data. Legesse (2000) also found that for most traits there was a significant correlation between hybrid performance and specific combining ability.

The suitability of yield potential, early and total yield, and quality traits for visual evaluation in pepper, combined with the difficulties encountered in replicated performance trials due to the immense number of measurements required, the need to reduce the breeding time, and economic considerations, pepper breeders generally use combined methods to discover lines with outstanding general and specific combining ability, methods that are better suited to the special nature of pepper plants and to the requirements of commercial breeding (Table 1).

In the combined crossing system, information on the general combining ability of homogeneous lines, preliminarily screened for individual plant performance, and on their specific combining ability in combinations is obtained from a single partial diallel cross based on the following considerations:

- All the combinations can be regarded as test combinations (they may theoretically produce the desired variety model)
- Groups of lines are formed for each main trait (earliness, high yield, large fruit, thick flesh, etc.) and each group is crossed with different testers
- Lines with better individual performance are crossed with a large number of testers
- New lines with excellent individual performance are included in the system prior to any knowledge of their general combining ability, generally in partial diallels involving earlier lines with known general combining ability
- Combinations are not made if they are unlikely to satisfy the variety model (e.g. between closely related lines, lines that do not complement each other, etc.).

The test combinations are screened visually in the first year, while those retained are screened in measured performance trials in the second and third years. In the third year, when the best lines are entered for state variety trials in the autumn, the best few combinations are tested at several locations by growers using various technologies.

This combined crossing system saves three years compared with the "maize model" and allows lines with good general combining ability and good specific combinations to be selected in a single step, while also allowing lines with relatively poor combining ability to be used in specific combinations (lines that would be rejected in the traditional scheme).

The majority of pepper breeding companies carry out accelerated testing in certain cases, for instance for combinations involving lines with outstanding individual performance, omitting the first, or even the first and second years of the testing scheme in the hope of rapidly developing a commercially successful hybrid.

Table 1

Schemes employed in the breeding of pepper hybrid varieties

Year	Line production generation	Variation 1	Variation 2	Variation 3	Variation 4	Variation 5
		Early testing (Standard)	Using homogeneous lines (Standard)	Using homogeneous lines (Combined system)	Using homogeneous lines (Special, accelerated procedure)	If the line production cross can also be taken as a test combination
1	Cross	Cross	Cross	Cross	Cross	Cross
2	F_1	F1	F1	F1	F1	Test combination F_1 (1st year: visual evaluation)
3	F_2	F2	F2	F2	F2	Test combination F_1 (2nd year: performance trial)
4	F_3	Test crosses (large volume)	F3	F3	F3	Test hybrid F_1 (performance trial)
5	F_4	Test combinations F_1 (performance trials)	F4	F4	F4	
6	F_5	Test combinations Crossing	Test crosses (normal volume)	Test crosses	Test crosses with selected lines	
7	etc.	Test combinations F_1 (performance trials)	Test combinations F_1 (performance trials)	Test combinations F_1 (1st year: visual evaluation)	Selected test combinations F_1 (performance trials)	
8		Test combinations F_1 (performance trials, 2nd year)	Test combinations Crossing	Test combinations F_1 (2nd year: performance trials)	Selected test hybrids F_1 (3rd year: performance trials)	
9		Test hybrid F_1 (performance trials)	Test combinations F_1 (performance trials)	Test hybrid F_1 (3rd year: performance trials)	Test hybrid F_1 (3rd year: performance trials)	
10		Test hybrid F_1 (performance trials, 2nd year)	Test combinations F_1 (performance trials, 2nd year)			
11			Test hybrid F_1 (performance trials)			
12			Test hybrid F_1 (performance trials, 2nd year)			

Even among the cross combinations developed for line production there may be some that satisfy the criteria of the variety model. These combinations are also treated as test combinations.

In order not to lose a year between the testing stages for each combination while F_1 seeds are being produced, sufficient seed must be produced from each cross for the next two testing stages.

Cross breeding

When cross-breeding pepper, one major economic decision that must be made by Hungarian breeders and breeding companies is where to conduct breeding experiments. Although pepper behaves as a self-pollinated plant from the breeding point of view, a small percentage of the plants will always be fertilised by alien pollen. Carrying out the experiments in an isolated system costs at least ten times as much as in field nurseries. As the funds available are limited, this means that ten times as much breeding stock can be planted in the field, but great care must be taken not to include spontaneous hybrids in further stages of testing. Even then it must be expected that the next generation will contain 1–2% alien material that could not be spotted phenotypically. Numerous pepper breeding companies in various parts of the world use field nurseries because reducing the breeding stock by 90% in isolated nurseries would be a greater loss than that represented by the 1–2% alien material in field stock, which will eventually be eliminated. In general the plants are sown in field nurseries from the F_2 until they are used in combinations or until a homogeneous line is developed, i.e. up till the F_4, F_5 or possibly F_6 generation, when the population size or number of lines is still large. In later phases of breeding isolated nurseries are generally used.

The aim of cross breeding may be to develop parental lines for the hybrid breeding discussed above or to develop constant varieties. Both of these aims can be achieved by cross breeding, with the differences outlined in Table 2. The methods applied in pepper breeding are single crosses, complex crossing series, the combination of either of these with the pedigree method, the backcross method, and the use of the doubled haploid (DH) method in connection with these. It is worth paying special attention to resistance breeding, the utilisation of male sterility and the role of molecular markers.

Table 2
Differences between pepper hybrid parental lines and constant varieties

Hybrid parental line	Constant variety
Homozygous pure line	Population in genetic equilibrium
Good general combining ability is a precondition (high proportion of additive gene effects)	General combining ability is not a precondition, but is generally adequate due to substantial additive gene effects
Good specific combining ability with another selected line	–
Deficient genome (requiring complementation by the other parental line)	Complete genome
Advantageous if it is a DH line	Not necessarily advantageous if it is a DH line

Breeding using single crosses

A single cross is sufficient if initial material that is relatively similar to the desired variety model and of commercial value is already available. Special types of single cross are the last cross in a crossing series and the last backcross if this involves a different recurrent parent.

In many cases the parents used in breeding crosses in pepper are not homozygous or even homogeneous, but segregating. This often necessitates selecting the parents for the desired traits before crossing is begun, carrying out the cross with a number of different plants, and planting the F_1 progeny of individual fruit from the cross in separate plots. A degree of segregation depending on the level of heterozygosity of the parents will be observed in these F_1 plants, so plants from which F_2 seed is to be saved must be labelled even in the F_1.

The number of crosses that must be made will depend on the homogeneity of the parents. If the parents are homozygous for all loci (e.g. DH) it is theoretically sufficient to make only one cross and select a single F_1 plant. For parents which are more or less heterozygous but where all the plants are uniform (e.g. F_1 hybrid variety), one cross is sufficient, but more F_1 plants are required, as the F_1 segregates. In the case of initial stock which is still segregating but where the traits required in crossing partners are manifested at a high level, it is often unnecessary to delay the cross for years until complete homozygosity is achieved. However, if such segregating parents are used, a larger number of plants must be included in crossing in order to increase the probability that positive combinations of genes for polygenic traits, for which the parents cannot yet be selected, will appear in the F_1, where selection can be carried out. If the parents are only thought to be homozygous, the possibility that segregation may occur in the F_1 for the above reasons should not be ignored.

The F_0 and F_1 generations of pepper breeding crosses (development of F_1 and F_2 seed) can be produced in a single year by sowing the parents in December and the F_1 in July.

The greater the proportion of homozygous loci in the parents and the lower the number of undesirable gene linkages that need to be broken, the smaller the plant number required in the F_2 generation. If the parents only differ for a single dominant gene (e.g. L3), in theory two F_2 plants would suffice, whereas for parents that differ for several polygenic traits with an unknown number of factors (e.g. disease tolerance, yield potential), even several hundred plants may not be enough to ensure that all the possible and expected combinations will appear.

In the progeny populations plant selection is carried out until the degree of homogeneity required by the experimental aims is reached.

If the model aimed at is a constant pepper variety, it can best be achieved using genetically balanced populations. Plant selection is thus continued until the differences between progeny plots with the level of homogeneity specified by the model are within the limits required by the model (approx. F_4, F_5). From then on the progeny populations that best match the variety model (e.g. the highest yielders) are selected and the genetic variation of the populations is reduced in the required direction by positive or negative selection, or perhaps by selecting new mother plants, as long as significant selection gain can be detected (approx. F_7–F_{10}).

Under ideal conditions, the parents of F_1 hybrid varieties are homozygous lines. The level of homogeneity required before lines can be included in test combinations can be achieved by the F_4 generation for lines where the genomes of the parents exhibit a high percentage of similarity, but in other cases not until the F_5 or F_6. From the practical point of view, lines in the F_7–F_8 generation are regarded as homozygous. Individual plant selection is therefore continued until this stage, but this means a large number of progeny plots, so F_4–F_6 progeny generations with a level of homogeneity (and value) acceptable for inclusion in test combinations can be retained as lines, subject to strict negative selection and homogeneity checks. After testing, lines with perfect homogeneity can be tested further as lines, while those that are not yet homogeneous must be subjected to further plant selection. Fresh seed of combinations that have reached the last performance trial prior to entry in official state trials (representing the seed that will be used for the official trials) is produced from parental lines maintained by means of negative selection in the second year of testing (F_6 at the earliest, generally F_7–F_8, but F_7–F_{10} for lines subjected to renewed plant selection).

Crossing series

It often happens in pepper breeding that the desired trait is only to be found in initial stock phenotypically very different from the variety model. In such cases the aim can only be achieved after several crossing cycles. If a single monogenic trait is to be incorporated from the diverse pepper type, the backcross method is most promising, but in the case of one or more polygenic traits, before each cross in the crossing series it is necessary to find suitable plants, or lines created from these, in the progeny population of the previous cross. This generally requires plant selection in large progeny populations, at least until the desired trait reaches the homogeneous state, so the following cross may not be possible until the F_5–F_7. In the case of polygenic pathogen tolerance, such as tolerance of CMV, *Xanthomonas* or *Verticillium*, finding plants with satisfactory tolerance in the segregating generation is complicated by the fact that selection requires natural infection, as no laboratory test methods are yet available for the detection of disease tolerance. Infection-free years, which are favourable for cultivation, slow down this selection process. However, soils in Moldavia have a homogeneous level of natural infection with the pathogens *Verticillium* and *Fusarium*, making selection efficient. The variety *Táltos*, for instance, was marketed in Hungary after three years of selection in Moldavia.

Greenleaf et al. (1969) gave a detailed description of the genealogy of the pepper variety Bighart, covering 36 generations and involving crosses with six pepper varieties. It is interesting to note that the Hungarian tomato-shaped pepper variety Greygo appears to have been developed from a population of Bighart selected for this shape by Canadian Hungarians, so this could be the origin of the unique L2 gene apparently carried by Greygo (Salamon, 2001).

An example of a crossing series is presented in Figure 3. Crossing series may be part of breeding to satisfy new market demands, in which case the intermediate initial stocks are mostly varieties in themselves. In other cases the crossing series is planned in advance and involves crosses at two or three levels, where the intermediate crossing partners are not usually varieties. In the example shown in the figure (the hybrid variety Apollo) it is clear that the whole pedigree is a crossing series designed to follow changes in market demands. Certain parts of the pedigree were planned, over the course of a few crosses, but these stages only lasted until the aim was achieved, in the form of lines with an improvement in the given trait. The crossing series finally resulting in the Apollo F_1 really lasted from 1972 (from the Antibois × Soroksári cross) to 1997 (to the test cross using the parental lines of Apollo). A total of 22 parents were involved in the 14 crosses carried out at six crossing levels. Nine of the lines developed at various crossing levels and used for further crossing also became commercial varieties (in order of development: Cecei édes, Csokros felálló, Javított Cecei, Fehérözön, Soroksári, Táltos, Hosszú Táltos, Syn. Cecei, Boni), while six were trait-enhancing intermediate lines. Five foreign varieties and two wild types were also used in the crossing series.

Crossing series are an essential tool for breeding strategies designed to produce competitive varieties incorporating new traits.

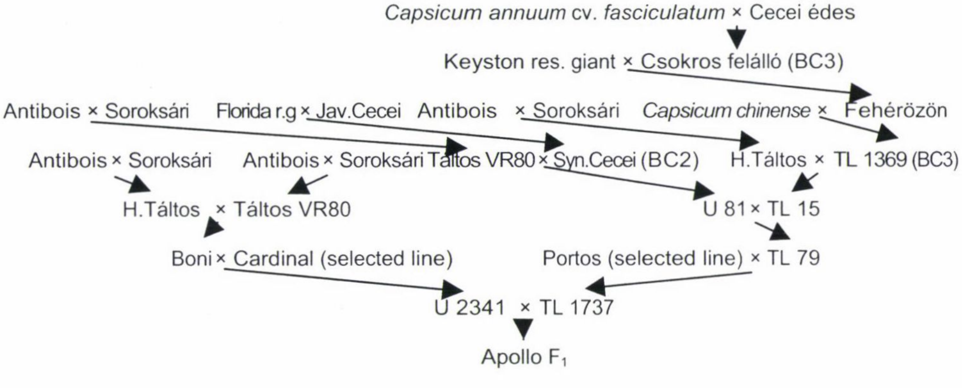

Fig. 3. Example of a crossing series: the pedigree of Apollo F_1

Backcross

The monogenic resistance of pepper and certain other monogenic traits are incorporated into the variety model by means of backcrossing. After selection for the desired trait, the following backcross is carried out on BC F_1 plants in the case of dominant traits and on BC F_2 plants for recessive traits.

Strictly speaking, the same recurrent parent should be used for all the backcrosses in a series, leading to the development of an isogenic line differing from the recurrent parent only for the incorporated gene. In pepper breeding practice, however, the recurrent parent may be changed during the backcrossing process for a number of reasons, most frequently because the original recurrent parent has lost its commercial importance, or because more valuable lines have become available in the meantime. The basic cross in the backcross process, particularly if it is an interspecific cross, only needs to be successfully achieved with a single *C. annuum* genotype, after which backcrosses can be continued with various types of varieties. For some variety models, especially if they only differ for a few traits, it is sufficient to direct the backcross programme towards these traits in the last or the last few backcrosses, for example to achieve a rapidly developing variety with medium-sized fruit, or a large-fruited variety with a moderate rate of development. If valuable breeding material is available that contains important monogenic traits (*L* genes, *ms, fa,* etc.) it is sufficient to use these as recurrent parents in the final stage(s) of backcrossing (unless these traits were the subject of the backcross), thus avoiding testing in earlier BC generations.

Plants homozygous for the incorporated trait, selected from the F_2 of a backcross carried out using the same recurrent parent throughout, can also be regarded as homozygous, or at least homogeneous for other traits. When the recurrent parent is changed during the backcross process, homogeneous lines can be selected in the third or fourth generation after the last change (in F_{3-4} after one BC, and in F_{2-3} after two BCs).

In practice the backcross is generally incomplete, because if a BC generation becomes phenotypically identical with the recurrent parent, or satisfies market demands, the backcross process is no longer continued. It may also be discontinued in the hope of retaining traits from the donor parent, particularly if it was a wild species or type, which may favourably complement the genome and broaden genetic variability.

In addition to the desired traits, it is worth carrying out selection in the BC F_1 (or in the F_2 when transferring recessive traits) for other traits or economic indices before carrying out further backcrossing. In pepper breeding very diverse plants are obtained in various backcross generations, so the population used for backcrossing needs to be relatively large if more rapid progress and a smaller number of backcrosses is to be achieved, and the plants to be used in crosses should be selected on the basis of yield and other indices. The importance of selecting the BC generations increases with the number of backcrosses. Even selection for a monogenic trait may be more complicated that simple testing based on a yes – no response. In most cases, if monogenic resistance is to function properly, a satisfactory genetic background is required, since various modifying factors may influence the manifestation of the gene (Szarka and Csilléry, 2001). For instance, a qualitative difference can be made between the hypersensitive symptoms indicative of the presence of the *Bs2* and *gds* genes, and even of the TMV resistance genes, depending on whether or not the plant has a satisfactory genetic background.

One argument put forward by those who oppose selection during backcrossing is that many favourable traits of the donor, which cannot be selected phenotypically, may be lost if selection is continually made for the phenotype of the recurrent parent.

It can be seen from these differing opinions on the technical details of backcrossing that in practical breeding the aim of the backcrossing process is not simply to produce a line theoretically isogenic for a single incorporated gene, but to develop a genotype that inherits from the donor not only the desired gene, but also other genome sections responsible for positive traits, and that can be regarded as isogenic in practice, taking into consideration the circumstances outlined above.

Doubled haploid (DH) lines

The advantages of DH lines, which are homozygous at all loci (Venczel and Mitykó, 1996; Venczel and Gémesné, 1998; Gyulai et al., 2000; Gémes Juhász et al., 2002), can be exploited most efficiently in pepper hybrid breeding.

The F_1 of homozygous parents that differ greatly from each other genetically (differing alleles at a high percentage of loci) can be expected to exhibit a high degree of heterozygosity, while the plants are complete homogeneous. The development of parental lines that can be regarded as homozygous for important traits suitable for selection is only possible with the traditional pedigree method after selection up to the F_7 or F_8 generation. Homozygosity for genes that are not manifested phenotypically, and which make up the larger part of the genome, will not, however, be reached until much later. Using the doubled haploid technique, on the other hand, homozygous lines can be obtained from the F_2 and all later DH generations.

Due to the high cost of DH line development, breeders would like to improve the probability that the DH lines developed from segregating generations will have the desired genotype. In this instance, however, patience is required. The first stage at which DH lines can be obtained is the F_1 of the line development cross. However, considering the fact that 20–50 combinations may be tested in order to achieve a certain variety model, the probability that the DH F_2 line obtained from any randomly selected F_1 donor plant will have the desired genotype is approximately 1:1000. Since the most favourable genotype is rarely manifested in the F_2, it is clear that the production of a DH F_3 generation will also be required. The probability that a DH F_3 line obtained from any F_2 donor plant randomly selected from the F_2 of F_1 material already selected for both the value of the combination and within the combination will have the desired genotype is again approximately 1:1000. If no selection is made in the F_1, this probability may drop to 1:10,000. The probability that DH plants with the desired genotype will be obtained can be enhanced by preliminary testing of the donor generations. DH F_2 plants are produced from the reserve seeds of F_1 plants selected on the basis of F_2 preliminary testing by means of traditional multiplication and selection for the desired phenotype. In the same way, DH F_3 plants are best produced from the reserve seed of F_2 plants preliminary selected for segregation in the F_3. A year is lost through the preliminary testing of the donor generation, but the ratio of valuable genotypes in the DH lines is greatly improved.

Perhaps too little emphasis is placed on the fact that one great advantage of using DH lines is the possibility of fixing genotypes that appear in the F_2 or F_3 but then segregate again when using traditional methods. Although these may theoretically reappear in later homozygous generations, in practice the likelihood of this happening is extremely small (in the same way, there is no chance that a perfect replication of the Apollo variety illustrated in Figure 3 will be produced at a later stage of the crossing series).

When a large number of DH lines are obtained from homogeneous, constant pepper varieties (or lines), it has been observed that these lines exhibit differences far greater than those seen in the original variety, while it may happen that none of the DH lines fits the original variety description. In addition, due to the manifestation of recessive genes, the majority of the lines are of less value than the original variety. This phenomenon must be expected to a certain extent even for DH plants obtained from breeding materials, but this is an important advantage of the DH technique rather than a disadvantage. The homozygous lines allow factors that are only lethal in the homozygous state, but also detract from the value of the variety in the heterozygous state, to be eliminated from the breeding material. A further advantage is that "braking factors" with an additive effect, which are not lethal but cause a loss of value when present in the homozygous state, can also be eliminated by rejecting the DH lines in which they are manifested. Plants from crossing populations developed using DH lines as parents give a better response to the DH technique, resulting in the induction of a larger number of haploid embryos. This can be attributed not only to selection for haploid induction ability, but also to the elimination of DH lines carrying lethal or negative traits.

Resistance breeding

In pepper breeding aimed at improving yield and yield stability, the incorporation of resistance to diseases and abiotic stress may have a greater effect than selection for yield components (Poulos, 1994). In addition, increasing importance is attached to the restriction of pesticide application. The types of resistance that are most important under Hungarian conditions, many of which cannot be achieved by chemical control, are listed in Table 3, which also lists when the disease or stress was last encountered.

Plants or lines with a satisfactory level of resistance or tolerance can only be selected from the breeding material against a background of 100%, homogeneous infection.

In the case of monogenic resistance this can only be achieved by artificial inoculation, especially as, in the case of natural infection, the elimination of the attacking pathogen usually takes place in the plant without any visible symptoms of the typical hypersensitive (resistant) response (lesion, leaf amputation).

Table 3

Pepper resistance genes of importance under Hungarian conditions

Pest or pathogen	Gene symbol	Gene effect	Recent occurrence	References
TMV	L_1	Dominant	Fehérözön, HRF (Tm0)	Holmes, 1934; Zatykó, 1979
	L_2	Dominant	Greygo (Tm1)	Greenleaf et el, 1969; Salamon et al., 2001
	L_3	Dominant	Ciklon, Cecil (Tm2)	Rast, 1977; Boukema, 1977; 1980
	L_4	Dominant	Century (Tm3)	Boukema, 1982; Csilléry, Ruskó, 1983; Sági, Salamon, 1998.
TSWV	Tsw	Dominant	PI 159236	Aleixandre et al., 2002
CMV	Tolerance	Polygenic	Táltos, Start	Pochard, Breuils, 1965; Marchoux et al., 1974
Xanthomonas campestris pv. *vesicatoria*	Bs1	Dominant		Cook, Stall, 1963
	Bs2	Dominant	ZKI 2349	Szarka, Csilléry, 2001
	Bs3	Dominant		
	gds	Recessive		Szarka, Csilléry, 1995; 2001
	Tolerance	Polygenic	Start	Zatykó, 1996
Verticillium and *Fusarium* species	Tolerance	Polygenic	Lasztocska, Táltos	
Nematode, *Meloidogyne incognita*	N	Dominant		Hare, 1937
Spider mite, *Tetranichus urticae*	Tolerance	Polygenic	Fehérözön	Zatykó, Martinovich, 1986

Plants exhibiting a resistant response after artificial inoculation are either selected for further breeding (in a backcross, in the case of a very low ratio of resistant plants, or in the early stages of resistance breeding, when the presence of the resistance gene in question is still at a critically low level in the breeding stock) or the inoculation results are simply noted as a test result that is taken into consideration as an important trait in later complex evaluation. In the latter case a large number of plants can be handled and the date of resistance testing can be made independent of the growing period, thus allowing the selection of genotypes that are not resistant but are outstanding for other valuable traits. During plant selection in the nursery, those with homozygous resistance can be identified on the basis of previous testing of the mother plants. To reduce costs, lines that are homogeneous for other traits but heterozygous for resistance are not made homozygous for resistance (by means of mother plant selection or with the aid of molecular markers) until later stages of selection. If the resistance breeding material is handled according to the testing scheme, resistance testing is not carried out in the F_1 or F_2 in the case of dominant resistance genes.

The efficient control of a given pathogen, particularly if it has a tendency to mutate, can be achieved more durably and over a wider race spectrum through the incorporation of tolerance. This takes considerably longer than that of monogenic resistance, but the number of factors responsible for tolerance and thus the degree of tolerance can be continually increased during a crossing series and any stage in the incorporation process may result in varieties suitable for cultivation. In the 1970s the CMV tolerance of the French variety Antibois was incorporated into varieties such as Táltos, while the CMV and *Xanthomonas* tolerance of *C. annuum* cv. Perennial was later introduced into Star and ZKI 1075 F_1.

Selection for tolerance is generally only possible in the case of natural infection, as tolerant plants do not give a hypersensitive reaction and are classified as susceptible according to the evaluation nomenclature of artificial inoculation. A clearer manifestation of differences in tolerance between the genotypes can perhaps be achieved by simulating natural infection or by providing conditions favourable to the pathogen.

Male sterility

The cytoplasmic male sterile pepper genotypes found or developed up till now have not been used in breeding due to the lack of restorers (Csilléry, in Somos, 1985).

Among the numerous alleles or genes causing genic sterility (ms), *ms-509*, produced by Pochard (1967) from a haploid line using a mutagen, and *ms-3*, another mutant obtained by Daskalov (1974) using gamma irradiation, have been most widely used in breeding. Of these, many breeders prefer *ms-3* due to its better seed yield in hybrid seed production and the easier identification of male sterile flowers.

The recessive *ms* gene is introduced into the female lines of the hybrid variety model by backcrossing, and progeny from *ms/ms* × *ms/ms+*, male sterile × fertile heterozygous crosses need to be constantly available for the production of hybrid seed (Moór, 1983; 1986; Moór and Csillléry, 1989). This material contains 50% male sterile and 50% fertile plants, and since no trait linked to the *ms* genes has yet been discovered, fertile plants must be eliminated on the basis of the characteristic male sterile flower or anther form prior to crossing. Despite this unavoidable elimination of 50% of the plants, an increasing number of hybrids are developed by pepper breeding companies on genic male-sterile female plants, because not only is castration and labelling unnecessary, but a perfect hybrid ratio is also guaranteed.

Utilisation of molecular markers

In practical pepper breeding non-gene-specific microsatellite or other markers are used to check the seed quality of hybrid varieties (by determining the hybrid %), to map genetic distances between lines and in selection for line maintenance, while specific gene markers are used for the selection of a few monogenically controlled traits in the cotyledon stage.

The seed control of hybrid varieties is traditionally carried out on the basis of a recessive trait introduced into the female line and scorable in the young seedling stage. These traits are generally controlled by an *al* (anthocyanin-less) or *ru* (rugose) marker gene. If the male line contains monogenic resistance (L_3, L_4) the presence of the recessive, susceptible female gene in the hybrid can also be determined by artificial inoculation. In earlier years, for lack of incorporated recessive marker genes, the hybrid % could only be determined by growing the hybrid if its phenotype differed from that of the female line. These traditional control techniques substantially limited the number of combinations that breeders could usefully develop, resulting in higher breeding costs and problems with breeder's rights. If hybrid seed is checked using molecular markers, considerably more line combinations can be made, while breeding capacity can be concentrated on the achievement of the variety model, rather than on the technical conditions required for quality control (incorporation of the *al* gene).

During the maintenance of homozygous lines the effect of open pollination with pollen from other pepper genotypes with a similar phenotype is difficult to avoid and may not become obvious. However, testing with several markers is capable of reliably eliminating plants with different sequences.

The use of gene markers has brought the greatest change in the enhancement of selection efficiency and the shortening of the breeding process. These are particularly important in the case of traits that can only be selected in plants bearing fruit, such as pungency or male sterility, or for selecting plants homozygous for dominant traits from the segregating generations with the help of codominant markers. This saves a year in the last phase of incorporating virus resistance genes. With the help of markers backcrosses can be carried out in the BC F_1 generation during the incorporation of recessive genes. Markers that can be used in practical pepper breeding have so far been developed mainly for resistance genes, but some companies treat these sequences as trade secrets.

References

Ahmed, N., Muzaraf, H. (2000): Heterosis studies for fruit yield and some economic characters in sweet pepper (*Capsicum annuum* L.). *Capsicum and Eggplant Newsletter*, **19**, 74–77.

Aleixandre, S. S., Niclós, J. D., Ripollés, S. R. (2002): *Genetical improvement of the resistance to the tomato spotted wilt virus (TSWV) in peppers*. Research Staff Publications. Universidad Politecnica de Valentia.

Allard, R. W. (1960): *Principles of Plant Breeding*. John Wiley and Sons, Inc., New York – London.

Banga, S. S., Banga, S. K. (1998): *Hybrid Cultivar Development*. Narosa Publishing House, New Delhi.

Boukema, I. W. (1977): Resistance in *Capsicum* to a pepper strain of TMV. "Capsicum 77", III. Meeting, Montfavet. pp. 85–88.

Boukema, I. W. (1980): Allelism of genes controlling resistance to TMV in *Capsicum* L. *Euphytica*, **29**, 433–439.

Boukema, I. W. (1982): Resistance to a new strain of TMV in *Capsicum chacoense* HUNZ. *Capsicum Newsletter*, **1**, 49–51.

Breuils, G., Pochard, E. (1975): Essai de fabrication de l'hybride de piment "Lamuyo-INRA" avec utilisation d'une stérilité male génique (ms-509). *Ann. Amélior. Pl.*, **25**, 399–409.

Chang, K. Y., Han, K. S., Ko, M. S. (1968a): Studies on the selection in red pepper breeding. 1. Variances and heritabilities. *Journal of the Institute for Agricultural Resources Utilization, Chinju Agricultural College*, **2**, 1–4.

Chang, K. Y., Han, K. S., Ko, M. S. (1968b): Studies on the selection in red pepper breeding. 2. Genotypic correlations. *Journal of the Institute for Agricultural Resources Utilization, Chinju Agricultural College*, **2**, 5–8.

Chang, W. N. (1977): Studies on genetic behaviour and breeding of sweet pepper. 3. A diallel analysis of plant height, plant weight, number of branch, total number of flowers, days to first flower and days to first fruit maturity. *Chinese Society for Horticultural Science, Journal*, **23**, 237–246.

Chang, W. N., Lin, K. J., Tseng, F. S. (1977): Studies on genetic behaviour and breeding of sweet pepper. 1. Heritability and correlation of quantitative characters. *Chinese Society for Horticultural Science, Journal*, **23**, 70–76.

Chang, W. N., Chung, W. J. (1979): Studies on genetic behaviour and breeding of sweet pepper. 4. Early generation heterosis and inbreeding depression. *NCHU Horticulture*, **4**, 9–12.

Choi, S. H., Kim, Y. C. (1986): The inheritance of anatomical components in red pepper. *Kyungpook National University, Agricultural Research Bulletin*, **4**, 12–17.

Chung, W. J. (1981): Studies on genetic behaviour and breeding of sweet pepper. 6. Path analysis of quantitative characters. *Taichung District Agricultural Improvement Station, Bulletin* **5**, 30–35.

Cook, A. A., Stall, R. E. (1963): Inheritance of resistance in pepper to bacterial spot. *Phytopathology*, **53**, 1060–1062.

Csilléry, G., Ruskó, J. (1983): Single lesion technique for the purpose of identification of the alleles on the L locus in *Capsicum*. *V. Capsicum Meeting*, Plovdiv, pp. 81–83.

Daskalov, S. (1974): Investigations on induced mutants in sweet pepper (*Capsicum annuum* L.). Eucarpia. *Genetics and Breeding of* Capsicum. Budapest, 1–4 July 1974, pp. 81–90.

Daskalov, S., Milkova, L. (1991): Induced mutations used in heterosis breeding of pepper (*Capsicum annuum* L.). Plant mutation breeding for crop improvement. *Proceeding of Akad. Inst. Genet.*, Sofia, pp. 499–504.

Depestre, T., Gomez, O., Espinosa, J. (1986): Genetic traits in pepper (*Capsicum annuum*). *Ciencia y Tecnica en la Agricultura: Hortalizas, Papa, Granos y Fibras*, **5**, 7–23.

Depestre, T. (1988): Heritability studies in sweet pepper. *Agrotechnica de Cuba*, **20**, 115–118.

Depestre, T., Gomez, O., Espinosa, J. (1989): Components of variability, heritability and genetic advance in pepper. *Ciencia y Tecnica en la Agricultura: Hortalizas, Papa, Granos y Fibras*, **8**, 91–95.

Doshi, K. M., Shukla, P. T. (2000a): Combining ability analysis for fresh fruit yield and its components over environments in chilli (*Capsicum annuum* L.). *Capsicum and Eggplant Newsletter,* **19**, 82–85.

Doshi, K. M., Shukla, P. T. (2000b): Genetics of yield and its components in chilli (*Capsicum annuum* L.). *Capsicum and Eggplant Newsletter*, **19**, 78–81.

Doshi, K. M., Shukla, M. R., Kathiria, K. B. (2001): Seedling analysis for the prediction of heterosis and combining ability (*Capsicum annuum* L.). *Capsicum and Eggplant Newsletter,* **20**, 46–49.

Gémes Juhász, A., Gajdos, L., Venczel, G., Sági, Z., Zatykó, L., Vági, P., Kristóf, Z. (2002): Production of doubled haploid breeding lines of pepper, eggplant, cucumber, zucchini and onion species. *Int. Conf. On Vegetables*, November 11–14, 2002, Bangalore. India. Abstracts, p. 151.

Gyulai, G., Gémesné, J. A., Sági, Z., Venczel, G., Pintér, P., Kristóf, Z., Törjék, O., Heszky, L., Bottka, S., Kiss, J., Zatykó, L. (2000): Doubled haploid and PCR-analysis of F_1 hybrid derived DH-R2 paprika (*Capsicum annuum* L.) lines. *J. Plant Physiol.*, **156**, 168–174.

Greenleaf, W. H., Hollingworth, M. H., Harris, H., Rymal, K. S. (1969): Bighart, an improved pimiento pepper (*Capsicum amnnuum* L.) variety. *HortScience*, **4**, 334–338.

Greenleaf, W. H. (1975): The Tabasco story. *HortScience, **10**. 2. 98.*

Hare, W. W. (1937): Inheritance of resistance to root-knot nematodes in pepper. *Phytopathology*, **47**, 455–459.

Holmes, F. O. (1934): Inheritance of ability to localize tobacco mosaic virus. *Phytopathology,* **24**, 984–1002.

Kalloo (1988): *Vegetable Breeding*. Volume I. CRC Press Inc., Boca Raton, Florida, USA

Kaul, B. L., Sharma, P. P. (1988): Heterosis and combining ability studies for some fruit characters in bell pepper (*Capsicum annuum* L.). *Vegetable Science*, **15**, 171–180.

Kuckuck, H., Kobabe, G., Wenzel, G. (1985): Grundzüge der Pflanzenzüchtung. Walter de Gruyter, Berlin – New York.

Legesse, G. (2000): Combining ability study for green fruit yield and its components in hot pepper (*Capsicum annuum* L.). *Acta Agron. Hung.*, **48**, 373–380.

Marchoux, G., Marrou, J., Quiot, J. B. (1974): Virologie vegetale. Complementation entre ARN de differentes souches du virus de la Mosaique du Concombre. Mise en evidence d'une interaction entre deux ARN pour determiner un type de symptome. *C. R. Acad. Sc. Paris,* 1943–1946.

Moór, A. (1983): The use of marker gene for assessing outcrossing ratio of pepper varieties. *Capsicum and Eggplant '83*. Vth Meeting, Plovdiv. pp. 71–76.

Moór, A. (1986): Hybrid seed production by male sterile female lines in Hungary. VIth Eucarpia *Meeting on Genetics and Breeding on Capsicum and Eggplant*, Zaragoza. pp. 51–54.

Moór, A., Csilléry, G. (1989): Hibridpaprika-magtermesztés hímsteril anyavonalakkal. (Hybrid paprika seed production on male sterile female lines.) *Zöldségtermesztési Kutató Intézet Bulletinje*, Kecskemét, **22**, 69–72.

Pochard, E., Breuils, G. (1965): La resistance du piment (*Capsicum annuum* L.) a la mosaique du tabac et au virus du concombre. Modalitées et transmission hereditaire. *J. Phyt. Phytopharm.*, Marseilles, pp. 189–193.

Pochard, E. (1967): *Poivron. Extrait du Rapport Annuel* 1967. INRA, Station d' Amelioration des Plantes Maraicheres, Montfavet.

Poulos, J. M. 1994: Pepper breeding (*Capsicum* spp.): achievements, challenges and possibilities. *Plant Breeding Abstracts,* **64**, 143–155.

Rast, A. T. B. (1977): Introductory remarks on strains of TMV infecting peppers in the Netherlands. *Proceedings Third Capsicum Eucarpia Meeting*, 5–8 July 1977.

Salamon, P., Venczel, G., Zatykó, L., Sági, Z. (2001): Studies on the Tobamovirus resistance of the pepper (*Capsicum annuum* L.) cultivar Greygo. *Int. Jour. of Horticultural Science*, **7**, 71–75.

Sági, Z., Salamon, P. (1998): Breeding white, sweet pepper hybrids armed with the tobamovirus resistance allele L^4. *Proceeding of the Xth Eucarpia Meeting on Genetics and Breeding on Capsicum and Eggplant,* Avignon (France), September 7–11, 1998, p. 173.

Sifriss, C., Sacks, J. M. (1980): The effect of distance between parents on the yield of sweet pepper × hot pepper hybrids, *Capsicum annuum* L. in a single harvest. *Theor. Appl. Genet.*, **58**, 253–256.

Singh, J., Ahmed, N., Virk, D. S. (1982): Inheritance of some quantitative characters in chilli pepper (*Capsicum annuum* L.). 1. Fruit yield, number and size. *Capsicum Newsletter*, **1**, 30.

Somos, A. (1985): A paprika, *Capsicum annuum* L. (Pepper, *Capsicum annuum* L.), *Magyarország Kultúrflórája* **54**, Akadémiai Kiadó. Budapest.

Szarka, J., Csilléry, G. (1995): Defense system against *Xanthomonas campestris* pv. *vesicatoria*. Eucarpia IX. *Meeting on Genetics and Breeding on Capsicum and Eggplant*. Budapest. pp. 184–187.

Szarka, J., Csilléry, G. (2001): General defense in the plant kingdom II. *Int. J. Hort. Sci*. Budapest, **7**, 73–77.

Venczel, G., Mitykó, J. (1996): Utilisation of androgenic doubled haploid (DH) lines in the improvement of an open pollinated pepper (*Capsicum annuum* L.) variety. *Horticultural Science,* **28**, 14–18.

Venczel, G., Gémesné, J. A. (1998): Pepper breeding methods and strategies related with *in vitro* haploid research. *Proceedings of the X. Eucarpia Meeting on Genetics and Breeding on Capsicum and Eggplant*, Avignon (France). pp. 96–97.

Zatykó, L. (1979): *Paprikatermesztés*. (Pepper production.) Mezőgazdasági Kiadó, Budapest.

Zatykó, L. (1979–1980): A dohány mozaik vírus (TMV) rezisztens paprikafajták viselkedése különböző körülmények között bekövetkezett fertőzés hatására. (Behaviour of paprika varieties resistant to tobacco mosaic virus (TMV) in response to infection under various conditions.) *ZKI Bulletin, Kecskemét*, **14**, 5–11.

Zatykó, L., Martinovich, V. (1986): Resistance to the red spider mite (*Tetranichus urticae* Koch.) in the "Fehérözön Synthetic" pepper variety. *VII. Eucarpia Meeting Capsicum and Eggplant*, Saragosa.

Zatykó, L., Sasvári, M. (1992): Moderation of the negative correlation between size and growth rate in new pepper varieties for consumption. Capsicum Newsletter (Special Issue). *VIII. Eucarpia Meeting, Genetics and Breeding on Capsicum and Eggplant*. Rome. pp. 100–105.

Zatykó, L. (1996): The breeding of pepper (*Capsicum annuum* L.) varieties resistant to bacterial spot, *Xanthomonas campestris* pv. *vesicatoria*. *Hungarian Agricultural Research*, pp. 8–10.

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Review

IMPROVEMENT IN THE HAPLOID TECHNIQUE ROUTINELY USED FOR BREEDING SWEET AND SPICE PEPPERS IN HUNGARY

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Large numbers of genetically stable, homozygous plants are needed for classical and molecular breeding programmes. *In vitro* anther culture has proved to be a useful tool for haploid/doubled haploid (DH) induction in pepper (*Capsicum annuum* L.) for more than twenty years. The present paper reports on a great improvement in the *in vitro* haploid induction and genome duplication methods routinely used for resistance breeding in sweet and spice peppers by two Hungarian research institutions, the Agricultural Biotechnology Center in Gödöllő and the Budapest Research Unit of the Vegetable Crops Research Institute.

As a result of the colchicine-stimulated early genome induction method, the critically low (<0.1%) regeneration frequency of spice pepper types became ten times greater, reaching a value of around 1.0%, though this was still considerably lower than that achieved in pepper varieties for fresh consumption (5–10%). Moreover, the ratio of useful doubled haploids was far higher (H:DH = 1:2 or 1:4) in some cases after colchicine treatment than that of untreated control plants (H:DH = 2:1 or 3:1, depending on the genotype).

An efficient method with good reproducibility, requiring less manual work, was elaborated for the *in vitro* genome duplication of pepper haploid regenerants using colchicine.

When the haploid induction ability of plants conventionally cultured in the greenhouse was compared to that of plants raised under artificial conditions in phytotron chambers (satisfactory day and night temperatures, illumination, humidity), the responsiveness of the latter microspores (ratio of plant regeneration) was found to be almost twice as high. The application of 3% maltose for six days at 35°C resulted in a 1.45% increase in the ratio of responding anthers and a 0.34% increase in plant regeneration, averaged over all the variety types. Phenosafranin staining was used for the analysis of microspore viability. The reduction in viability during the induction period proved to be less pronounced in lines with better androgenetic responses than in those with poorer responsiveness.

Key words: haploid, doubled haploid, anther culture, colchicine, chromosome doubling, pepper, *Capsicum annuum* L.

Importance of the induced haploid method in breeding

The spontaneous occurrence of haploids in nature was first discovered several decades ago (e.g. twin-embryo haploids), and their induction under experimental conditions opens up a whole range of possibilities. Haploid plants containing a single chromosome set usually arise spontaneously, without fertilisation, from the egg-cell, from some other haploid cell of the female gametophyte (e.g. the synergids) or from male gametes (parthenogenesis, apomixis).

Over the last twenty years investigations on the application of the *in vitro* haploid technique based on the artificial induction of gametes has become a major research field in plant biotechnology. In the course of *in vitro* haploid induction, artificially induced stress effects (e.g. heat shock, chemical or physical treatment) are employed to influence the natural processes of development and differentiation in the male and female gametes. Stress may divert gamete development from the gametophytic developmental pathway to the sporophytic pathway, resulting in the development of androgenetic or gynogenetic embryos or morphogenic callus. Plants arising from gametes carry the genetic material of a single parent, so they can be regarded as genetically identical, or homozygous. The sporophytic development pathway starting from immature male gametes is known as *in vitro* androgenesis, and that starting from female gametes as *in vitro* gynogenesis. In many plant species, *in vitro* androgenesis can be induced from microspore, pollen or anther cultures. In the case of gynogenesis, embryos and plant regeneration can usually be achieved by culturing unfertilised ovaries, or more precisely, embryo sacs (ovules).

The vast majority of plants regenerated after *in vitro* andro- or gynogenesis have a haploid genetic complement, but plants with other ploidy levels may also arise spontaneously, most frequently "spontaneous" diploids. These plants also develop in most cases from a single micro- or macrospore, but during the early stages of *in vitro* culturing "spontaneous" chromosome doubling (endoreduplication) or cell nucleus fusion leads to the doubling of the genome. These plants can be regarded as doubled haploids. While haploid plants have no value in breeding due to their sterility, they are excellent material for genetic analysis. Valuable doubled haploid plants can be obtained through the *in vivo* or *in vitro* diploidisation of the genome of haploid plants.

The practical application of the *in vitro* haploid method has numerous advantages. In Hungary, as in many other countries, new varieties can only be state registered or included in the EU variety list if they possess distinctness, uniformity and stability (DUS) in their phenotypic traits, i.e. the variety, or the parental lines of an F_1 hybrid, should be largely homozygous. The market also expects varieties to have homogeneous qualitative and quantitative traits. Using the *in vitro* haploid technique, genetic combinations (meiotic recombinations) can be fixed within a single generation, producing perfectly homozygous breeding stock. This is particularly important for the fixation of types within segregating generations. In the case of traditional breeding, valuable recombinations may be lost when the segregating generations segregate further. If these fixed recombinations produce genotypes that can be entered directly for state trials or used in further breeding, a great deal of time and money can be saved. By fixing promising recombinations between genetically distant parents, selected lines can be used in test hybrid combinations in the next generation. The greater the number of fixed genetic combinations, the greater the likelihood that the best gene combinations will be found for variety development. Using traditional breeding methods, obtaining pure forms of dominant traits is a difficult process, as homozygotes cannot be distinguished phenotypically from heterozygotes. The improvement of polygenic traits is even more complicated. The phenotype of haploid and doubled haploid plants is a clear reflection of the genotype, thus assisting the breeder to make an evaluation. One outstanding advantage of the *in vitro* haploid technique is that when major traits are determined by recessive genes, combinations carrying these genes can be fixed in the homozygous state within a generation. A further advantage of the method is that the genome can be freed of alleles that are sublethal in the homozygous state. This is of particular importance in the case of open-pollinated species. The genome of self-pollinated species carries fewer such alleles due to selection pressure. Haploid or doubled haploid plants developed using the haploid technique form valuable basic material not only for breeders, but also for functional genetic analysis on allele interactions and on the combining ability of alleles, for the genetic mapping of individual species, for molecular breeding using genetic markers and for genetic transformations.

Large numbers of genetically stable, homozygous plants are needed for breeding programmes. If a sufficient quantity of doubled haploid plants is to be developed for breeding, the efficiency of *in vitro* haploid methods must be constantly improved. Efficient genome duplication methods will also be required for the production of a large volume of fertile breeding material.

Historical review of the development of haploid induction methods for pepper

The first report on the classical development of haploid pepper *Capsicum annuum* L. lines was published by Christensen and Bamford (1943), who considered these to be spontaneous haploids arising from twin embryos. A decade later, Morgan and Rappleye (1954) stated that spontaneous twin-embryo haploids were probably of maternal origin, arising from the synergids. These early reports suggested that twin embryos occurred at a frequency of 0.01–0.1%. The first haploid plant of androgenetic origin was reported by Campos and Morgan (1958). Experiments on twin-embryo haploids were later continued by French breeders, who succeeded in inducing haploids by treating flower buds with N_2O, thus increasing the haploid frequency from 0.02% to 0.2% (Dumas de

Vaulx and Pochard, 1974). Twin-embryo haploids were found with similar frequency (0.01–0.2%) by Pochard and Dumas de Vaulx (1971) during selection in the F_2 generation of a Yolo Wonder × B 107 cross. These were rediploidised using colchicine treatment and the homozygous lines obtained were compared with the parents and the original F_1 and F_5 generations. Although the yield average of certain doubled haploid lines approached that of the F_1, these lines matured later and had much poorer fertility. Work on genotype selection made it clear that the efficiency of breeding efforts was influenced to a great extent by the strong dependence of parthenogenetic haploid development on the maternal genotype and on the environment (Pochard and Dumas de Vaulx, 1979). Despite the difficulties, the classical, induction and genotype selection methods outlined above opened the way for the utilisation of haploids in breeding practice.

The next milestone in the use of haploids was the elaboration of *in vitro* anther culture techniques in the early 1970s. This provided breeders with a much more efficient laboratory method. Research teams in China (Wang et al., 1973) and India (George and Narayanaswamy, 1973) were the first to report successful plant regeneration from pepper anther cultures, i.e. from haploid embryos, and these were followed by numerous other publications (Kuo et al., 1973; Saccardo and Devreux, 1974; Novák, 1974; Harn et al., 1975). These papers dealt mainly with haploid callus and/or embryo induction, and rarely reported successful plant regeneration. Sibi et al. (1979) improved the reproducibility and efficiency of anther culture through the cold treatment of flower buds (+4°C, 48 h), the determination of the optimum microspore stage (pollen mitosis I) and the development of specific nutrient combinations. In this way 1–3 plants were obtained from 100 isolated anthers. However, the most efficient method proved to be the technique developed by Dumas de Vaulx et al. (1981), who exposed isolated anthers to heat shock at +35°C in the dark for 8 days prior to culturing. With this technique, 5–10% plant regeneration could be achieved for the pepper genotypes tested, thus allowing haploids to be used in practice. Experimentation aimed at optimising the protocol was continued in many laboratories. Vagera and Havránek (1983) experimented with the addition of charcoal, Abak (1983) with a high sucrose concentration, Morrison et al. (1986) with a two-phase nutrient combination containing charcoal, and Munyon et al. (1989) with treatment at +29°C in continuous light. However, none of them succeeded in improving the efficiency of the method described by Dumas de Vaulx et al. (1981), so this technique is still used in the majority of laboratories, possibly with some modification to the specific environment.

Cytological and morphological analyses on pepper lines of androgenetic origin demonstrated that, in addition to haploid regenerants, diploid and triploid plants also arose at various frequencies, together with chimeras (haplodiploids) (Sibi et al., 1979; Dumas de Vaulx et al., 1981; Vagera and Havránek, 1985). A detailed review of the results achieved up till 1990 was published by Vagera (1990).

In recent years, in addition to the practical application of doubled haploids, emphasis has been placed on the refinement of the environmental conditions for anther culturing. It was found that the optimisation of the environmental factors influencing the development of anther donor plants (soil quality, irrigation, humidity, illumination intensity and duration, temperature, nutrient replacement, plant protection) improved the response of the plants. Kristiansen and Andersen (1993) reported that the androgenic response was greatly influenced by the age of the donor plants, the temperature and the photoperiod. In their experiments the ideal plant growth temperature was found to be 26°C. Those grown at 16°C had very poor embryo induction (0.2%), while growth at 30°C drastically reduced the response of donor plants. Ltifi and Wenczel (1994) raised plants at two temperatures (25°C and 10°C) and found that for two of three varieties the higher temperature gave the better result. It was concluded from the results that the optimum plant growth temperature was genotype-dependent.

In genotypes with a particularly poor response, the stimulation of embryo induction is extremely important. While Dumas de Vaulx et al. (1981) recommended treating anthers at 35°C for 8 days, Gonzalez-Melendi et al. (1996) suggested treating flower buds at 4°C for 2–4 days prior to exposing the anthers to heat treatment (37°C) or starvation (nutrient medium containing mannitol). An effective induction method was also reported by Dolcet-Sanjuan et al. (1997), who kept the anther cultures at 7°C for a week, after which they were maintained on maltose-enriched induction and regeneration medium at 28°C for eight weeks. This resulted in a high embryo induction frequency (0–3561 embryos/100 anthers), but unfortunately a significant proportion of the embryos exhibited abnormal development, and the plant regeneration percentage was only 0–8.3% for the genotypes tested. The positive effect of maltose (3%) and a higher kinetin concentration (0.2–0.3 mg/l) was also confirmed by Gémes Juhász et al. (1998a). In addition to the successful application of kinetin (0.1–0.5 mg/l), some authors have found another cytokinin, BAP (0.6 mg/l), to promote the androgenic response of some recalcitrant genotypes (Qin and Rotino, 1993).

As observed for other plant species, the successful induction of androgenesis in pepper depends not only on the growth conditions of the donor plants and the various anther culture techniques, but also on the genetic background. Results obtained in recent years suggest that, depending on the genotype, 0–75 plants can be regenerated per 100 anthers from *in vitro* pepper anther cultures (Sibi et al., 1979; Dumas de Vaulx et al., 1981; Vagera and Havránek, 1985; Fári, 1986; Morrison et al., 1986; Qin and Rotino, 1993; Kristiansen and Andersen, 1993; Ltifi and Wenczel, 1994; Mitykó et al., 1995a, b; Dolcet-Sanjuan et al., 1997; Gémes Juhász et al., 1997; 1998b; 2001c; 2002). The number of responding anthers (those where some extent of embryo development was observed) was often four to eight times as great as the number of regenerated plants.

Anther culture as a possible starting point for haploid induction

Anther culture involves the dissection of anthers containing pollen at the required stage of development, and the induction and maintenance of androgenesis on artificial nutrient medium under sterile, controlled conditions. The aim is to raise plants with a reduced ploidy level from gametes (pollen haploids) *in vitro*. Androgenesis and haploid plant regeneration are achieved by altering the developmental pathway of the uninuclear, vacuolated pollen. In normal cases, the sporophytic development of plants is completed when meiosis takes place in the microspore mother cells, leading to a tetrad containing four haploid, uninuclear microspores. The initially uninuclear microspores become vacuolated in the course of development. The appearance of the vacuoles is accompanied by the degradation of the original cytoplasm and of the organelles it contains, giving way to the development of a new cytoplasm with its own organelles. In the meantime the originally uninuclear cell undergoes asymmetric mitotic cell division, resulting in a large vegetative cell and a smaller generative cell. The vegetative cell no longer divides, its role being the regulation of pollen grain development, the formation of the pollen tube and the introduction of the sperm into the ovary. The generative cell undergoes a further S phase, and then remains in phase G2 until the sperms have developed. The mitotic cell division of the generative cell takes place either in the pollen grain (B-celled pollen) or later in the pollen tube (Z-celled pollen). At a certain stage of microgametogenesis, however, the pathway of further development is not yet determined, so the developmental direction of the gametophyte can be shifted towards the sporophytic development pathway. If uninuclear, vacuolated microspores in the first pollen mitosis stage, or anthers containing them, are isolated from the mother plant and cultured under artificial conditions, sporophytic division can be observed in the microspores after 1–2 days, leading to the development of multicellular pollen grains, the further differentiation of which leads to pollen embryos from which haploid plants can be directly raised (Dudits and Heszky, 1990).

Many papers have been published on the optimal stage of microspore development for the induction of microspore embryogenesis in pepper. Based on practical experience, Dumas de Vaulx et al. (1981) suggested that the late uninuclear stage of microspore development was the most suitable for induction. Ultrastructural analyses carried out by Gonzalez-Melendi et al. (1995; 1996) confirmed that the late uninuclear stage of microspore development was ideal for the induction of androgenesis in pepper, though in some lines microspores in the early binuclear stage could also be induced.

Isolated microspore culture as an alternative for haploid induction

So far, haploid induction has been most successful in anther culture both for sweet and spice pepper. However, anther culture requires a great deal of manual work, and the efficiency of the method is poorer than that of well-functioning isolated microspore cultures.

The first step in this latter technique is the surface sterilization of the collected flower buds. The exploration of the microspores is carried out by physical smashing, most often using a blender. The debris is filtered through a nylon filter with a mesh size slightly larger than the microspore diameter. The pellet is collected by centrifugation. After adjusting the optimal microspore density, the microspores are cultured in liquid culture medium. The culturing of the microspore/pollen embryos is similar to that in the anther culture method.

The routine application of microspore cultures would be a further step in the development of a haploid induction technology, opening up the way for selection and transformation at the microspore level.

Major results achieved in Hungary in the development of haploid lines of sweet and spice pepper

The first haploid pepper breeding programme in Hungary was set up in the Tissue Culture Laboratory of the Agricultural Biotechnology Center (ABC) in Gödöllő in the early 1990s, based on the successful anther culture technique elaborated by Dumas de Vaulx et al. (1981). New prospects were opened up at ABC by a short-term training course in the laboratory of Dumas de Vaulx (Station d'Amelioration des Plantes Maraicheres, Institut National de la Recherche Agronomique (INRA), Montfavet/France). The successful adaptation of the method was followed by the wide-range testing of numerous varieties, types, hybrids and breeding lines of sweet and spice peppers from Hungary and abroad in order to classify them on the basis of their androgenic response (Mitykó et al., 1995a). This project was started in collaboration with Miklós Fári, András Andrásfalvy and Gábor Csilléry. Besides investigations on many resistant breeding lines, various pepper species, interspecific hybrids and exotic pepper lines were also tested. These first achievements were followed by attempts to improve the efficiency of plant regeneration and to achieve results with recalcitrant genotypes. Although the modification of nutrient components and hormone concentrations did not lead to significant results in the case of recalcitrant genotypes, the use of colchicine to stimulate early genome induction proved successful. Isolated anthers were induced on modified Cp nutrient medium supplemented with colchicine (Mitykó et al., 1999), after which they were cultured according to the protocol of Dumas de Vaulx et al. (1981). The division processes taking place in the microspores were monitored on transmission electron microscope (TEM) sections for seven days after induction.

The technique is based on the fact that colchicine is an antimicrotubular drug leading to abnormal processes in the course of cell division. The plant cytoskeleton is constructed from microtubules, consisting mainly of tubulin, and microfilaments containing actin. These filamentary components are known to have numerous structural functions and to be involved in movement within the cell. The role of microtubules in meiotic and mitotic cell division is outstandingly important, since the fibres of the nuclear spindle developing during cell division consist of microtubules, the evolution and decomposition of which is an extremely labile process, sensitive to chemicals, irradiation and heat. The well-known effect of colchicine in inhibiting mitosis is based on the fact that, at a concentration of a few mM, it can bind to tubulin molecules and prevent them from polymerising.

In addition to the general mitosis-inhibiting effect of colchicine, it was reported by Zaki and Dickinson (1990; 1991; 1995) that colchicine treatment on the microspores of *Brassica* caused the microtubules to become slacker and realigned, thus allowing the cell nucleus to migrate towards the centre of the cell. As nuclei near the centre of the cell undergo symmetric division with greater frequency than nuclei close to the cell-wall, colchicine no doubt has an important role in the induction of symmetric divisions. In turn, symmetric microspore division has been proved to lead to a greater frequency of sporophytic division resulting in haploid embryo induction, while asymmetric division is generally characteristic of the gametophytic developmental pathway.

In the course of the studies a larger ratio of symmetric divisions was found in treated pepper microspores, and the microspores developing from these symmetric divisions more frequently developed into embryogenic structures from which haploid or doubled haploid plants could be regenerated (Mitykó et al., 1999). As a direct result of the early genome induction induced by colchicine, the critically low (<0.1%) regeneration frequency of spice pepper types became ten times greater, reaching a value of around 1.0%, though this was still considerably lower than that achieved for sweet pepper varieties (5–10%). A further advantage of the method is that there was a higher ratio of doubled haploid plants among the regenerants. While in the untreated control experiments the ratio of valuable doubled haploids (DH) was extremely low compared with the undesirable haploid (H) plants (H:DH = 2:1 or 3:1, depending on the genotype), this ratio was much more favourable after colchicine treatment, being at least 50% (H:DH = 1:1) and in some cases far higher (H:DH = 1:2 or 1:4).

The chromosome numbers of the first samples were counted in root tips stained with acetocarmine, as no flow cytometry equipment was available in Hungary. Later, the rapid determination of the chromosome number using a flow cytometer (PARTEC CA-II) became possible, first in Austria (ARC Seibersdorf Research GmbH), and then at the Department of Genetics, Kossuth Lajos University, Debrecen. Since 1995, the flow cytometric evaluation of regenerated plants has been carried out in collaboration with the Biotechnology Laboratory of the Budapest Research Unit of the Vegetable Crops Research Institute.

Plants in which genome duplication had not taken place were treated with colchicine in the 4–6-leaf stage. The plants were immersed in a solution containing 0.3% colchicine and 1% DMSO (dimethyl sulphoxide) for 3 hours in the dark, ensuring that the solution completely covered the shoot tips. After the removal of the colchicine solution the plants were rinsed three times with distilled water. As a result of the treatment the chromosome number was doubled in 80% of the haploid plants. Although there are certain risks involved in colchicine treatment, it has been demonstrated that this application rate and duration does not increase the ratio of chimeras or mixoploid plants, and there was no reduction in fertility. After planting in Jiffy peat cubes and careful acclimatisation, the plants were transferred to the greenhouse, where they were grown to maturity.

The experimental work underway in the Biotechnology Laboratory of the Budapest Research Unit of the Vegetable Crops Research Institute has focussed in recent years on improving the efficiency of the haploid technique. Since 1995 anther cultures have been started from anther donor plants from lines or F_1 hybrids of six types of Hungarian sweet pepper varieties (white Cecei, white blocky, light green blocky, dark green blocky, tomato-shaped, white apple-shaped), two Turkish types (Dolma, Charliston), three Spanish types (Dolce Italiano, Lamuyo, red blocky), three Dutch types (dark green blocky, light green blocky, Kapia), and from various spice pepper lines and F_1 hybrids.

The results achieved in recent years suggest that the growth conditions of the anther donor plants play a decisive role in determining the androgenetic responsiveness of the various genotypes. When the haploid induction ability of plants conventionally cultured in the greenhouse was compared with that of plants raised under optimum artificial conditions in phytotron chambers (satisfactory day and night temperatures, illumination, humidity), the responsiveness of the latter microspores (ratio of plant regeneration) proved to be almost twice as high. When the data were analysed for various types of varieties, plant regeneration was found to be best for the white apple-shaped and Cecei varieties and for the white or light green blocky types, while the lowest values were recorded for the dark green and tomato-shaped types (Gémes Juhász, 2001a). Changes in the viability of microspores when maintained in *in vitro* culture are also of significance from the point of view of successful androgenesis induction. The results indicate that the best way to analyse microspore viability in pepper is to apply phenosafranin staining. In dead microspores the red fluorescence originating from phenosafranin masked the weaker blue autofluorescence, so red fluorescence was indicative of dead microspores and blue autofluorescence of viable ones. Analyses on the viability of pepper microspores (Gémes Juhász and Kristóf, 1999b; 2001a; Gyulai et al., 2000) revealed a gradual loss of viability during anther culture. This reduction in viability during the induction period was less pronounced in lines with better androgenetic responses than in those with poorer responsiveness.

Prior to the initiation of *in vitro* cultures the closed flower buds were sterilised. Due to the bacterial infection observed in some experiments, the traditional sterilisation procedure was modified, as the usual treatment with 20% bleach was unable to provide complete protection against the bacterium strains attacking the buds. The ratio of infection was particularly high in the hot summer months. The best sterilisation technique proved to be the use of a 1% concentration of the disinfectant Dodenal (Merck/Arcana, Chem. Ph. Fabrik, GmbH, Austria), which gave approx. 100 times greater protection against saprophytic bacteria than the chemical previously applied (Gémes Juhász and Hevesi, 2000, Gémes Juhász 2001a). The multiplication of bacteria which survived even this form of sterilisation was inhibited by adding Cefotaxim (200 mg/l) to the induction medium. The use of antibiotics did not have any notable influence on embryo induction.

The induction medium was prepared as reported by Dumas de Vaulx et al. (1981). In order to improve the efficiency of microspore induction, investigations were made on the effect of maltose on the induction of microspore embryogenesis and plant regeneration. In the case of barley (Kuhlmann and Foroughi-Wehr, 1989; Finnie et al., 1989) and wheat (Zhou et al., 1991; Karsai et al., 1994) the use of maltose as a source of sugar led to an improvement in the *in vitro* androgenesis efficiency. Maltose is hydrolysed more slowly than sucrose in the medium, thus delaying the availability of glucose to the culture. This stress is thought to enhance the induction of androgenesis. Tests on the joint effect of maltose concentration and treatment duration on induction showed that when induction medium supplemented with 3% maltose was applied for six days at 35°C, there was a 1.45% increase in the ratio of responding anthers and a 0.34% increase in plant regeneration, averaged over all the variety types. Above-average increments were obtained for the white blocky and Cecei lines, which have a poorer anther response than the apple-shaped pepper type (Gémes Juhász, 2001a). The use of maltose during the induction phase also proved successful in the case of spice pepper (Gémes Juhász et al., 1999a).

Before plants originating from anther culture can be used for practical breeding purposes it is essential to determine the genome size of the plants, since only diploid (doubled haploid) plants are fertile and suitable for breeding. The genome size of regenerants was first determined in the seedling stage using a PARTEC I flow cytometer (Partec GmbH, Münster, Germany) equipped with a high pressure mercury vapour lamp (Osram HBO 100W/2). The nuclei were isolated from the samples using the method of Doležel et al. (1989). Leaf segments measuring approx. 0.5 cm^2 were placed in 1 ml LBO1 buffer and chopped minutely using a razor blade. A pure cell nucleus suspension was obtained by filtration through a 30 μm Ø Cell Trics/TM (Partec GmbH, Münster, Germany) membrane. Samples containing cell nuclei were labelled with 1 ml

DAPI solution (Partec High resolution Kit Type P, Solution A). The DNA content of each sample was measured in three replications. Control samples (cell nucleus isolates) were prepared from normal diploid plant leaves.

The genome size determination allowed haploid plants arising from anther culture to be clearly distinguished from those that had undergone spontaneous diploidisation. An efficient method with good reproducibility, requiring less manual work, was elaborated for the *in vitro* genome duplication of pepper haploids (Gémes Juhász et al., 2001a, b, c). Regenerant plants in the 2–3-leaf state, shown by flow cytometer analysis to be haploid, were placed on hormone-free medium containing 400 mg/l colchicine for two, four or six days. The effect of the treatment in inducing genome duplication was checked by genome size determinations on new shoots (leaves) developing after the treatment. Although the 4-day treatment significantly increased the number of doubled haploids compared with the 2-day treatment, still only 24.22% of the treated plants became diploidised, so neither treatment appeared to be sufficient. The high frequency (24.2%) of mixoploids containing both haploid and diploid cells indicated that genome duplication was not yet complete. When the treatment period was increased to six days, a further significant increase was obtained compared with the 4-day treatment. Depending on the genotype, the rate of rediploidisation was 67–100%. Colchicine treatment had no adverse effect on the survival of the plants. Although phenotypic mutations were only observed after colchicine treatment in a negligible number of doubled haploid regenerants over the course of five years, special attention must be paid to the possibility of mutants arising in the progeny populations.

Histological studies on pepper anther cultures drew attention to the fact that the *in vitro* induction treatments sometimes stimulated cell division not only in the microspores but also in somatic cells in the connective region of the anther wall (Horváth et al., 1999; Gémes Juhász, 2001a). Before using spontaneous diploids as breeding material it is therefore advisable to check that they are homozygous if the initial anther donor plants represent the F_1 generation, since spontaneous diploid plants of microspore origin will only contain an allele from one of the parents, while regenerants arising from somatic cells will carry alleles from both parents.

Practical application of haploid and doubled haploid plants/lines

The importance of haploids for practical application soon became obvious to both geneticists and breeders, and they are now an indispensable tool for molecular scientists. Starting from the trisomic analysis carried out by Pochard (1977), through their use in breeding programmes for the analysis and fixation of disease resistance and other resistance traits (Abak et al., 1982; Doré and Dumas de Vaulx, 1989; Daubèze et al., 1990), haploids have now reached the molecular genetics laboratories.

A research team in Montfavet (Lefebvre et al., 1995) has prepared an intraspecific linkage map for chili pepper, using RFLP and RAPD markers in segregating doubled haploid populations.

The induction of pollen embryogenesis is now being studied at cell level in pepper using *in situ* methods involving various types of DNA, RNA and protein probes (Gonzalez-Melendi et al., 1996; Bárány et al., 2001).

Long-term cooperation was established between the Agricultural Biotechnology Center (ABC) and three Hungarian pepper breeding companies, Budakert Ltd., represented by Gábor Csilléry, Primordium Ltd., represented by János Szarka, and the Kalocsa Red Pepper Research and Development Non-Profit Co., represented by Ferenc Márkus† and József Kapitány. Within the framework of this cooperation work has been underway for almost ten years on the fixation of genes encoding resistance to bacteria (*Xanthomonas vesicatoria*), viruses (TMV, PMMoV, CMV, TSWV) and nematodes (*Meloidogyne incognita, M. arenaria, M. javanica*) in homozygous form, and their incorporation into sweet peppers and spice pepper by means of *in vitro* anther culture.

Priority has been given by these breeders and scientists to wide-ranging investigations on various types of resistance and to their novel interpretation and characterisation. Doubled haploid (DH) lines have been used extremely successfully in this work. In addition to specific resistance genes encoding the hypersensitive reaction (HR) associated with rapid cell destruction, which are widely used in resistance breeding, general defence reactions (GDR) also exist, which are not pathogen-specific and which aim at saving cells attacked by pathogens at all cost. The stimulus threshold is much lower for GDR than for HR, while the reaction rate is higher. The two types of defence reactions are based on different strategies and have diverse biochemical backgrounds. GDR is able to act as a plant immune system, while HR is a specific defence aimed at excluding certain pathogens. In the case of pepper, GDR is regulated by the monogenic recessive gene *gds*, while the hypersensitive reaction to the bacterium *Xanthomonas vesicatoria* is controlled by the monogenic dominant gene *Bs-2*. Due to their functions, the effect of the two genes on each other does not result in the expected dominant-recessive relationship. The general and specific defence reactions complement each other to produce an integral system of plant disease resistance (Szarka and Csilléry, 1995; 2001; Csilléry et al., 2004a, b).

Important results have recently been obtained in the development or enhancing of resistance to bacterial and viral diseases and to various pests (nematodes) (Márkus et al., 2003; Csilléry et al., 2005).

The aim of further cooperation is the wide-ranging investigation of resistance traits and the development of new, resistant pepper varieties through a combination of conventional breeding and biotechnological methods. Some of the DH lines developed at ABC are in use within the institute as basic material for genetic research.

Over the last 50 years, the Vegetable Crops Research Institute has been the main developer of new varieties of sweet pepper in Hungary. Doubled haploids have been used as parental lines carrying resistance and important qualitative and quantitative traits in homozygous form (Gémes Juhász 1997; 1998a, b; 2001d; 2002; 2006a, b). Thanks to the work of the breeders Lajos Zatykó and Zsolt Sági and the variety maintainers Mrs J. Moór and Gizella Venczel, the vast majority of the breeding combinations currently used for sweet pepper have lines developed over the last 15 years from haploid induction as one or both of their parents, so an increasing proportion of the hybrids released in the future will have parents of doubled haploid origin.

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References

Abak, K., Pochard, E., Dumas de Vaulx, R. (1982): Transmission of resistance to *Phytophthora capsici* on roots and stems of pepper plants: study of DH lines issued from the cross "PM 217" × "Yolo Wonder" through anther culture. *Capsicum Newsletter*, **1**, 62–64.

Abak, K. (1983): Study on the anther culture *in vitro* of pepper (*Capsicum annuum* L.). *Capsicum Newsletter,* **2**, 72–73.

Ahmadian, P., Testillano, P., Gonzalez, P., Fadón, B., Prestamo, G., Jimenez-Duran, G., Risueno, M. C. (1998): Cell biology of the pollen developmental program and the induction of microspore embryogenesis in *Capsicum annuum* L. *Proceedings of the Xth Meeting on Genetics and Breeding of Capsicum and Eggplant* (Avignon, France Sept. 7–11). pp. 183–187.

Bárány, I., Testillano, P. S., Mitykó, J., Risueño, M. C. (2001): The switch of the microspore developmental program in Capsicum involves HSP70 expression and leads to the production of haploid plants. *Int. J. Dev. Biol. 45. Supplement,* **1**, 39–40.

Campos, F. F., Morgan, D. T. Jr. (1958): Haploid pepper from a sperm: an androgenic haploid of *Capsicum frutescens*. *J. Hered.*, **49**, 134–137.

Christensen, H. M., Bamford, R. (1943): Haploids in twin seedlings of pepper, *Capsicum annuum* L. *J. Hered.*, **34**, 99–104.

Csilléry, G., Szarka, E., Sárdi, É., Mitykó, J., Kapitány, J., Nagy, B., Szarka, J. (2004a): The unity of plant defense: Genetics, breeding and physiology. *XIIth EUCARPIA Meeting on Genetics and Breeding of* Capsicum *& Eggplant*. Noordwijkerhout - The Netherlands. May 17–19. pp. 147–153.

Csilléry, G., Szarka, E., Sárdi, É., Mitykó, J., Márkus, F., Kapitány, J., Nagy, B., Szarka, J. (2004b): A növényi betegségellenállóság egységes egésze. *X. Növénynemesítési Tudományos Napok*. Magyar Tudományos Akadémia Budapest, Február 18–19. p. 37.

Csilléry, G., Márkus, F., Balázs, E., Kiss, Gy. B., Kapitány, J., Mitykó, J., Szarka, E., Gutermuth, Á., Földi T., Szarka, J. (2005): Lehetőségek a paprika fonalféreg ellenállóságának fokozására. *XI. Növénynemesítési Tudományos Napok*. Magyar Tudományos Akadémia Budapest, Március 3–4. p. 67.

Daubèze, A. M., Palloix, A., Pochard, E. (1990): Resistance of androgenetic autodiploid lines of pepper to *Phytophtora capsici* and Tobacco Mosaic Virus under high temperature. *Capsicum Newsletter*, **8–9**, 47–48.

Doležel, J., Binarova, P., Lucretti, S. (1989): Analysis of nuclear DNA content in plant cells by flow citometry. *Biol plant.*, **31**, 113–120.

Doré, C., Dumas de Vaulx, R. (1989): Utilisation de l'haploïdie dans l'amélioration de quelques espèces potagères (asperge, chou, piment, aubergine et melon). Cinquantenaire de la Culture in vitro. Versailles (France) 24–25 Oct. Ed. INRA Paris, 1990: *Les colloques de l'INRA*, pp. 177–185.

Dudits, D., Heszky, L. (1990): *Növénybiotechnológia.* (Plant Biotechnology.) Mezőgazdasági Kiadó, Budapest. 310 p.

Dumas de Vaulx, R., Pochard, E. (1974): Essai d'introduction de la parthénogenèse haploïde par action du protoxyde d'azote sur les fleurs de piments (*Capsicum annuum*). *Ann. Amélior. Plantes*, **24**, 283–305.

Dumas de Vaulx, R., Chambonnet, D., Pochard, E. (1981): Culture *in vitro* d'anthères de Piment (*Capsicum annuum* L.): amélioration des taux d'obtention de plantes chez différents génotypes par des traitements à +35 °C. *Agronomie*, **1**, 859–864.

Fári, M. (1986): Pepper (*Capsicum annuum* L.). pp. 345–362. In: Bajaj, Y. P. S. (ed.), *Biotechnology in Agriculture and Forestry*. Vol.2. Crops I. Springer, Berlin, Heidelberg, New York.

Finnie, S. J., Powell, W., Dyer, A. F. (1989): The effect of carbohydrate composition and concentration on anther culture response in barley (*Hordeum vulgare* L.). *Plant Breeding*, **103**, 110–118.

Gémes Juhász, A., Martinovich, L., Venczel, G., Sági, Z., Somogyi, N., Zatykó, L. (1997): Kettőshaploid növények alkalmazása a paprika, a vöröshagyma és a cukkini nemesítésben. (Use of doubled haploid plants in the breeding of paprika, onion and courgette.) *Új Kertgazdaság*, **3**, 9–15.

Gémes Juhász, A., Sági, Z., Salamon, P., Somogyi, N., Zatykó, L., Venczel, G. (1998a): Experiences and results of *in vitro* haploid methods application in pepper breeding programme. *Xth EUCARPIA Meeting on Genetics and Breeding of* Capsicum *and Eggplant.* Avignon, France, September 7–11. Abstract pp. 201–203.

Gémes Juhász, A., Sági, Z., Salamon, P., Somogyi, N., Zatykó, L., Venczel, G. (1998b): L^4 rezisztencia gént hordozó homozigóta paprika nemesítési vonalak számának növelése DH technikával. (Use of the DH technique to increase the number of homozygous paprika breeding lines carrying the L^4 resistance gene.) "Vas Károly és Lippai János" tudományos ülésszak előadásai és poszterei. Budapest, 1998. szept. 16–18. *A Kertészeti és Élelmiszeripari Egyetem Kiadványai* **X**, p.462.

Gémes Juhász, A., Somogyi, N., Sági, Z., Venczel, G. (1999a): A maltóz fűszerpaprika androgenezisre gyakorolt hatása portokkultúrában. (Effect of maltose on the androgenesis of spice paprika in anther culture.) *Kertgazdaság*, **31/3**, 96–102.

Gémes Juhász, A., Kristóf, Z. (1999b): Is there any relationship between pollen viability and the pepper anther response ability? Workshop, Working Group 4. COST-ACTION 824 *Cellular and Molecular Aspects of Pollen Embryogenesis*. Prague, Czech Republic, 22–24 April. Abstract Book: 21.

Gémes Juhász, A., Hevesi, M. (2000): Paprika portokkultúra tenyészetek mentesítése a bakteriális fertőzésektől. (How to avoid bacterial infection in paprika anther cultures.) *Növénynemesítési Tudományos Napok 2000* MTA, Budapest, március 8–9. Poszterösszefoglaló, p. 83.

Gémes Juhász, A. (2001a): In vitro *androgenetikus és ginogenetikus módszerek alkalmazása haploid/dihaploid növények előállítására zöldségnövényeknél*. PhD értekezés. Szent István Egyetem, Kertészeti Doktori Iskola, Budapest.

Gémes Juhász, A., Petus, M., Venczel, G., Zatykó, L., Sági, Z. (2001b): Hatékony genomduplikációs eljárás kidolgozása a paprika portokkultúrából származó haploidok kezelésére. (Elaboration of an efficient genome duplication technique for treating haploids arising from paprika anther cultures.) *Növénynemesítési Tudományos Napok '01* MTA, Budapest 2001 január 23–24. Előadás és poszterösszefoglalók: p. 27.

Gémes Juhász, A., Petus, M., Venzel, G., Sági, Z. (2001c): Colchicine an efficient genome doubling agent for anther derived haploid paprika (*Capsicum annuum* L.) plants. Papers *Book of Xth Eucarpia Meeting on Genetics and Breeding of* Capsicum *and Eggplant*, Antalya, Turkey, 9–13 April.

Gémes Juhász, A., Gyulai, G., Petus, M., Venczel, G., Sági, Z., Zatykó, L. (2001d): DH-breeding of sweet pepper (*Capsicum annuum* L.). *Agriculture and Biotechnology*, Cost Action 824. Biotechnological approaches for utilisation of gametic cells. pp. 157–159.

Gémes Juhász, A., Gajdos, L., Venczel, G., Sági, Z., Zatykó, L., Vági, P., Kristóf, Z. (2002): Production of doubled haploid breeding lines in case of pepper, eggplant, cucumber, zucchini and onion species. *Proceeding of International Conference on Vegetables*, Bangalore, India, Nov. 11–14. p. 151.

Gémes Juhász, A., Vörösváry, B., Vági, P., Kristóf, Z. (2006a): Summarized results of *in vitro* haploid induction of vegetables as paprika, spice paprika, eggplant, cucumber, zucchini and onion. Proceeding of COST Action *851 Gametic Cells and Molecular Breeding for Crop Improvement,* Final Workshop, February 10–11, Vienna, Austria. p. 27.

Gémes Juhász, A (2006b): Doubled haploids as tools in Hungarian paprika breeding. *Proceeding of 18th International Pepper Conference*, Palm Springs, California, May 21–23. p. 7.

George, L., Narayanaswamy, S. (1973): Haploid Capsicum through experimental androgenesis. Brief report. *Protoplasma*, **78**, 467–470.

Gonzalez-Melendi, P., Testillano, P. S., Ahmadian, P., Fadon, B., Vicente, O., Risueno, M. C. (1995): *In situ* characterisation of the late vacuolate microspore as a convenient stage to induce embryogenesis in Capsicum. *Protoplasma*, **187**, 60–71.

Gonzalez-Melendi, P., Testillano, P. S., Ahmadian, P., Fadón, B., Risueño, M. C. (1996): New in situ approaches to study the induction of pollen embryogenesis in *Capsicum annuum* L. *Eur. J. Cell Biol.*, **69**, 373–386.

Gyulai, G., Gémesné Juhász, A., Sági, Z., Venzcel, G., Pintér, I., Kristóf, Z., Törjék, O., Heszky, L., Bottka, S., Kiss, J., Zatykó, L. (2000): Doubled haploid development and PCR-analysis of F_1 hybrid derived DH-R2 paprika (*Capsicum annuum* L.) lines. *J. Plant Physiol.*, **156**, 168–174.

Harn, C., Kim, M. Z., Choi, K. T., Lee, Y. I. (1975): Production of haploid callus and embryoid from the cultured anther of *Capsicum annuum*. *Sabrao J.*, **7**, 71–77.

Horváth, M., Gémesné Juhász, A., Kristóf, Z. (1999): Haploid indukció mikroszkópos vizsgálata paprika portokkultúrában. (Microscopic analysis of haploid induction in paprika anther cultures.) *X. Magyar Növényanatómiai Szimpózium*, Debrecen, 1999. augusztus 26–28. Poszterösszefoglaló, pp. 90–91.

Karsai, I., Bedő, Z., Hayes, P. M. (1994): Effect of induction medium pH and maltose concentration on *in vitro* androgenesis of hexaploid winter triticale and wheat. *Plant Cell Tissue Org. Cult.*, **39**, 49–55.

Kristiansen, K., Andersen, S. B. (1993): Effects of donor plant temperature, photoperiod and age on anther culture response of *Capsicum annuum* L., *Euphytica*, **67**, 105–109.

Kulhman, U., Foroughi-Wehr, B. (1989): Production of doubled haploid lines in frequencies sufficient for barley breeding programs. *Plant Cell Rep.*, **8**, 78–81.

Kuo, J. S., Wang, Y. Y., Chien, N. F., Ku, S. J., Kung, L., Hsu, H. C. (1973): Investigations on the anther culture *in vitro* of *Nicotiana tabacum* L. and *Capsicum annuum* L. *Acta Bot. Sinica*, **15**, 43–47.

Lefebvre, V., Palloix, A., Caranta, C., Pochard, E. (1995): Construction of an intraspecific integrated linkage map of pepper using molecular markers and doubled-haploid progenies. *Genome*, **38**, 112–121.

Ltifi, A., Wenczel, G. (1994): Anther culture of hot and sweet pepper (*Capsicum annuum* L.): Influence of genotype and plant growth temperature. *Capsicum and Eggplant Newsletter*, **13**, 74–77.

Márkus, F., Kapitány, J., Mitykó, J., Csilléry, G., Szarka, J., Somogyi, G., (2003): A fűszerpaprika betegségellenállóság mint élelmiszerbiztonságot meghatározó tényező. *IX. Növénynemesítési Tudományos Napok.* Magyar Tudományos Akadémia Budapest, Március 5–6. p. 46.

Mitykó, J., Andrásfalvy, A., Csilléry, G., Fári, M. (1995a): Anther culture response in different genotypes and F_1 hybrids of pepper (*Capsicum annuum* L.). *Plant Breeding*, **114**, 78–80.

Mitykó, J., Chambonnet, D., Ádám, G., Andrásfalvy, A., Fári, M. (1995b): In vitro haploidy of spice and bell peppers: its control for large-scale application. *IXth Meeting on Genetics and Plant Breeding on* Capsicum *and Eggplant*. Budapest. August 21–25. pp. 64–66.

Mitykó, J., Szabó, L., Barnabás, B. (1999): Colchicine induced ultrastructural changes in barley and pepper microspores. *The Third International Symposium in the Series: Recent Advances in Plant Biotechnology*, September 4–10. Stara Lesna, Slovakia. *Biologia*, **54,** 24–25.

Morgan, D. T., Rappleye, R. D. (1954): A cytogenetic study on the origin of multiple seedlings of *Capsicum frutescens*. *Amer. J. Bot.*, **41**, 576–585.

Morrison, R. A., Koning, R. E., Evans, D. A. (1986): Anther culture of an interspecific hybrid of Capsicum. *J. Plant Physiology*, **126**, 1–9.

Munyon, I. P., Hubstenberger, J. F., Phillips, G. C. (1989): Origin of plantlets and callus obtained from chile pepper anther cultures. *In Vitro Cellular and Developmental Biology*, **25**, 293–296.

Novák, F. J. (1974): Induction of a haploid callus in anther culture of *Capsicum* sp. *Z. Pflanzenzücht.*, **72**, 46–54.

Pickering, R. A., Devaux, P. (1992): Haploid production: approaches and use in plant breeding. In: Shewry, P. R. (ed.), *Barley: Genetics, Biochemistry, Molecular Biology and Biotechnology*. Alden Press, Oxford. pp. 519–547.

Pochard, E., Dumas de Vaulx, R. (1971): La monoploïdie chez le piment (*Capsicum annuum* L.). Réalisation pratique d'un cycle de sélection accéléré par passage à l'état monoploïde en troisième génération. *Z. Pflanzenzüchtung*, **65**, 23–46.

Pochard, E. (1977): Localization of genes in *Capsicum annuum* L. by trisomic analysis. *Ann. Amélior. Plantes*, **27**, 255–266.

Pochard, E., Dumas de Vaulx, R. (1979): Haploid parthenogenesis in *Capsicum annuum* L. *The Biology and Taxonomy of the* Solanaceae. Linnean Soc. Symp. Birmingham (GBR) Ser. 7. pp. 455–472.

Qin, X., Rotino, G. L. (1993): Anther culture of several sweet and hot pepper genotypes. *Capsicum and Eggplant Newsletter*, **12**, 59–62.

Saccardo, F., Devreux, M. (1974): *In vitro* production of plantlets from anther culture of *Capsicum annuum*. *Genetics and Breeding of* Capsicum. II. Eucarpia Meeting, Budapest. pp. 45–50.

Sibi, M., Dumas de Vaulx, R., Chambonnet, D. (1979): Obtention de plantes haploïdes par androgenése in vitro chez le Piment (*Capsicum annuum* L.). *Ann. Amélior. Plantes*, **29**, 583–606.

Szarka, J., Csilléry, G. (1995): Defence system against *Xanthomonas campestris* pv. *vesicatoria* in pepper. *IXth EUCARPIA Meeting on Genetics and Breeding on* Capsicum *and Eggplant*. Budapest, Hungary, August 21–25. pp 184–187.

Szarka, J., Csilléry, G. (2001): General defense system in the plant kingdom. *Int. J. Hort. Sci.*, **7**, 79–84.

Testillano, P. S., Gonzalez-Melendi, P., Fadon, B., Vicente, O., Risueno M. C. (1994): New in situ approaches to study the induction of pollen embryogenesis in *Capsicum.* In: Heberle-Bors, E., Hesse, M., Vicente, O. (eds.), *Frontiers in Sexual Plant Reproduction Research.* Univ. Vienna, Vienna, p. 16.

Vagera, J. (1990): Pepper (*Capsicum* spp.): In vitro Induction of Haploids. pp. 374–392. In: Bajaj, Y. P. S. (ed.), *Biotechnology in Agriculture and Forestry*, Vol. 12. Haploids in Crop Improvement I. Springer-Verlag Berlin – Heidelberg

Vagera, J., Havránek, P. (1983): Stimulating effect of activated charcoal in the induction of in vitro androgenesis in *Capsicum annuum* L. *Capsicum Newsletters,* **2**, 63–65.

Vagera, J., Havránek, P. (1985): *In vitro* induction of androgenesis in *Capsicum annuum* L. and its genetic aspects. *Biol. Plant.,* **27**, 10–21.

Wang, Y., Sun, C., Wang, C., Chien, N. (1973): The induction of the pollen plants of *Triticale* and *Capsicum annuum* from anther culture. *Scientia Sinica*, **16**, 147–151.

Zaki, M. A. M., Dickinson, H. G. (1990): Structural changes during the first divisions of embryos resulting from anther and microspore culture in *Brassica napus*. *Protoplasma*, **156**, 149–162.

Zaki, M. A. M., Dickinson, H. G. (1991): Microspore-derived embryos in *Brassica:* the significance of division symmetry in pollen mitosis I to embryogenic development. *Sex Plant Reprod.*, **4**, 48–55.

Zaki, M. A. M., Dickinson, H. G. (1995): Modification of cell development *in vitro*: The effect of colchicine on anther and isolated microspore culture in *Brassica napus*. *Plant Cell, Tissue and Organ Culture*, **40**, 255–270.

Zhou, C., Zheng, Y., Konzak, C. I. (1991): Several medium components affecting albinism in wheat anther culture. *Plant Cell Rep.*, **10**, 63–69.

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Review

GENERAL DEFENSE REACTION IN THE PLANT KINGDOM

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The fact that production is often unsuccessful even when resistant varieties are selected on the basis of the hypersensitive reaction can be attributed to the lack of adequate knowledge on plant disease resistance. In addition to specific plant responses to pathogen species, plants also possess an aspecific defense reaction which, instead of causing rapid tissue destruction, is based on the opposite strategy, protecting the plant against attack by microbes through tissue compaction achieved by cell enlargement and cell division.

Genetic analyses carried out in pepper revealed that the general defense reaction was inherited as a monogenic recessive trait (*gds*). Pathophysiological observations indicate that the stimulus threshold is lower and the reaction rate faster than for specific defense reactions. Biochemical analyses suggest that, unlike plants exhibiting rapid tissue destruction, plants containing the *gds* gene do not require an oxidative burst elicited by hydrogen peroxide to stimulate the defense mechanism. It was also found that the regulation of the general defense system involves metabolic pathways that are independent of salicylic acid. The general and specific plant reactions form an integrated system of plant defense.

Key words: hypersensitive reaction, general defense reaction, pepper, cell destruction, cell division, hydrogen peroxide (H_2O_2), salicylic acid (SA)

Introduction

Scientists studying the relationship between plants and pathogens progress from the specific to the general. The problems this involves were soon encountered by resistance breeders attempting to restore the defense system that protects cultivated plants from pathogens. Breeding for resistance against pathogen species inducing easily diagnosed, specific symptoms has now been underway for several decades and has been based on plant responses manifested in the form of rapid tissue destruction, i.e. the hypersensitive reaction (HR). However, the phenotypic manifestation of the HR may be induced by many different kinds of pathophysiological processes. Consequently, in order to avoid misunderstanding, in the present work, within the concept of HR, only specific, phenotypically perceptible plant responses induced by a specific resistance gene are referred to as resistance responses.

Specific defense reactions to a single pathogen species proved to be insufficient in the long term, as summarised by Flor (1955) in the "gene for gene" theory, which states that the resistance of resistant plants can only be broken down as the result of selection pressure on the pathogen population. Due to the breakdown of resistance, susceptible symptoms indicative of disease are observed instead of the rapid tissue destruction associated with the resistant response. One weakness of this hypothesis is that it does not take into account the general defense reaction that has proved to be a fundamental attribute of the plants forming the basis of the host–pathogen relationship. The discovery of the general defense reaction made a great contribution to a more complex understanding of how plants respond to microbes and pathogens (Szarka and Csilléry, 1995). The most important characteristic of the general defense reaction is that, instead of the rapid destruction of cells and tissues, it aims to save plant parts affected by biotic or abiotic stress at any cost (Szarka and Csilléry, 2001a, b). This response is manifested as tissue compaction arising as the result of cell enlargement and cell division (Szarka et al., 2002; Csilléry et al., 2004).

Symptomological and pathological observations

Tissue alterations developing under natural conditions as the result of the general defense reaction, susceptible symptoms and the specific defense reaction were investigated using the response of bean to infection with *Pseudomonas phaseolicola* as an example (Fig. 1). The adaxial leaf surface in a bean line with no resistance genes specific to the *P. phaseolicola* bacterium, but with a high-level general defense reaction, exhibited a crater-like indentation at the site of infection, while the tissue protruded on the abaxial surface. On the leaves of susceptible bean plants, the spread of the bacterium was indicated by a concentric water-soaked patch around the site of infection. The functioning of a specific resistance gene providing resistance to the *P. phaseolicola* bacterium resulted in the tissues around the site of infection becoming purple and dry.

The identification of the three reaction types and their relationship to each other were further investigated by electron microscope analysis on another host–pathogen relationship: pepper – *Xanthomonas vesicatoria* bacterium (Fig. 2). The parenchyma cells in the leaf blades of a pepper line containing the *gds* gene responsible for a high-level general defense reaction became substantially larger and divided, causing a protuberance on the abaxial surface (Fig. 2a). The area around the site of infection subsided and died on the leaves of susceptible plants, and was surrounded by a ring of enlarged parenchyma cells (Fig. 2b). Leaves of plants containing the specific *Bs-3* gene exhibited a great extent of rapid destruction; the cells dried out and the tissues shrank. There was no cell enlargement in the area surrounding the lesion (Fig. 2c).

The incorporation of genes encoding the specific or the high-level general defense reaction into a single plant facilitated the analysis of the interaction between defense systems that use different strategies. For this purpose the recessive *gds* gene, encoding a high-level general defense reaction, and the dominant *Bs-2* gene, originating from the *Capsicum chacoense* species and causing intense purple tissue coloration during the localisation of the pathogen, were incorporated into the same plant. Four pepper lines were developed, one susceptible to the *Xanthomonas vesicatoria* bacterium, one containing the *Bs-2* specific resistance gene, one containing the *gds* gene and one containing both genes in the homozygous constellation (Fig. 3).

In response to infiltration with a *X. vesicatoria* suspension with a concentration of 10^8 cells/ml, the affected leaf tissue of the susceptible line rapidly dried as a consequence of over-infection (Fig. 3a).

On plants carrying the specific resistance gene *Bs-2*, the central part of the inoculated leaf tissue exhibited rapid destruction and also dried due to over-infection. However, the edge of the infiltrated patch turned purple, indicating the presence of an active defense reaction, i.e. the functioning of the *Bs-2* gene (Fig. 3b). It is thus clear that the response controlled by the specific resistance gene does not necessarily involve tissue destruction. The tissue destruction resulting from over-infection and the tissue alterations controlled by the specific resistance gene have a different physiological background due to the passive or active role of the host plant. In the case of over-infection, the specific resistance gene is not activated.

As the result of the intense general defense reaction provided by the *gds* gene, instead of undergoing destruction, the leaf tissue at the site of inoculation was compacted and thickened by cell division (Fig. 3c). This indicates that the hypersensitive tissue destruction caused by phytopathogenic *Pseudomonas* species in incompatible interactions is not a generally valid phenomenon (Klement et al., 1964).

In plants containing both the *gds* and *Bs-2* genes in the homozygous constellation, the inoculated leaf tissue compacted and thickened in a manner characteristic of the general defense reaction. The presence of the *Bs-2* gene could only be implied from the purple coloration of tissues along the veins, where the defense reaction is less pronounced. This tissue alteration serves as proof of the fact that the general defense reaction regulated by the recessive *gds* gene is far more rapid than the process encoded by the dominant specific gene *Bs-2* (Fig. 3d). The fundamental importance of the general defense system is confirmed by the fact that a monogenic, recessive gene encoding a more rapid physiological process was manifested rather than a monogenic, dominant specific resistance gene, a phenomenon never previously recorded in plant genetics.

The interaction between the general and specific defense reactions and its relationship to the susceptible reaction was further investigated on the basis of the response to stresses of various intensity.

The leaves of *Nicotiana tabacum* cv. Pallagi were infiltrated with 10^1–10^9 cells/ml concentrations of the bacteria *Pseudomonas tabaci* and *P. phaseolicola* (Fig. 4).

Treatment with inocula containing 10^1–10^7 cells/ml *P. tabaci* or 10^1–10^8 cells/ml *P. phaseolicola* caused a slight protuberance on the adaxial leaf surface and a chlorotic patch without a sharply defined edge, indicating that under optimum environmental conditions the general defense system of tobacco was able to tolerate the stress induced by 10^7 (*P. tabaci*) or 10^8 (*P. phaseolicola*) cells/ml of bacterium suspension without tissue destruction. Under unfavourable environmental conditions the fundamental life processes of the plants, including the general defense system, are inhibited, leading to greater exposure, poorer stress tolerance and, in the case of a susceptible host–pathogen relationship, disease symptoms.

When applied at a concentration of 10^8 cells/ml, *P. tabaci* caused characteristic disease symptoms; the infected tissues became water-soaked and were surrounded by a slightly chlorotic region. The general defense reaction is activated by all types of biotic stress. If it is not effective, the microbe, now acting as a pathogen, is able to evade the general defense system of the plant, now becoming a host plant, and a susceptible host–pathogen relationship evolves. This relationship is a delicately balanced state depending on the stress caused by the pathogen and the general defense ability of the plant.

Irrespective of the compatible or incompatible interaction, at a concentration of 10^9 cells/ml both bacterium species led to rapid tissue destruction. This allows conclusions to be drawn not on the two bacterium species, but on tobacco, the general defense system of which was not able to tolerate this unnatural over-infection.

The only way that plants could escape the susceptible host–pathogen relationship evolving due to the inefficiency of the general defense reaction was to evolve a specific defense reaction to eliminate the specific stimulus caused by the pathogen. Knowing the differences in the reaction rates of the general and specific defense reactions, tests were made with *Xanthomonas vesicatoria* inocula with concentrations of 10^1–10^9 cells/ml in order to determine the stimulus threshold required for the two reactions to be activated. The tests were carried out on the leaves of three pepper lines, one susceptible, one containing the gene responsible for the specific defense reaction (*Bs-2*), and one containing the *gds* gene responsible for the general defense reaction (Fig. 5).

In susceptible pepper plants and in those containing the specific resistance gene (*Bs-2*), leaf sections infiltrated with 10^1–10^7 cell/ml concentrations of *Xanthomonas vesicatoria* exhibited mild chlorosis and slight protuberances due to a slight enlargement of the leaf cells, indicating an incomplete general defense system (Fig. 5a). A suspension containing 10^8 bacterium cells/ml caused halos on the susceptible pepper line (Fig. 5b), while purple patches that did not dry were observed on plants containing the *Bs-2* resistance gene (Fig. 5c), i.e. specific resistance symptoms. As near-isogenic pepper lines were used in the experiments, it can be concluded that the specific resistance gene starts functioning in the stress range where the incomplete general defense system of

In order to justify the use of the term "general" in the designation general defense reaction, specific resistance genes against fungal, bacterial and viral pathogens were incorporated into pepper lines together with the *gds* gene (Fig. 6).

Infection with the pathogens *Leveillula taurica* (Fig. 6a) and *X. vesicatoria*, which exhibit a compatible interaction with pepper, and *X. phaseoli, P. phaseolicola* and *P. fluorescens* (Fig. 6b), where there is an incompatible interaction, led to the tissue alterations characteristic of the *gds* gene. Even the typical symptoms of tobacco mosaic virus (TMV) appearing in plants containing the resistance gene *L3* (Fig. 6d) were unable to develop in the presence of the *gds* gene (Fig. 6c) when the fully developed leaves were inoculated using the carborundum method. Electron microscope analysis revealed that the cells around the wounds caused by the carborundum grit became enlarged and began to divide. It seems likely that the virus was localised as a side-effect of the reaction to mechanical injury. This response to mechanical injury indicates that the *gds* gene is also effective against abiotic stresses.

In view of the general defense reaction, the selection method used during resistance breeding, based on rapid tissue destruction, should be reconsidered, since this type of selection leads to a great deterioration in the general defense ability of the plants. Against a background of a seriously impaired general defense system, genes providing specific resistance to individual pathogens are unable to function satisfactorily.

Biochemical studies

In addition to the pathological and genetic characterisation of the general and specific defense reactions, a number of biochemical characteristics of these plant reactions were also investigated.

The biochemical processes forming the background of rapid tissue destruction, i.e. the hypersensitive reaction (HR), have been studied for a long time. However, several authors have reported that the examination of biochemical processes also reveals the local and systemic effects of the destruction of tissues directly affected by the pathogen. Localised acquired resistance (LAR) occurs within a narrow zone around the HR lesion, and is characterized by the strong stimulation of the enzymatic and non-enzymatic components of the plant defense system (Dorey et al., 1997; 1998). Systemic acquired resistance (SAR) develops in non-infected parts of the plant and only triggers a subset of defense reactions (Ryals et al., 1996). The biochemical processes observed in plants showing rapid tissue destruction are highly diverse, and are regulated by different signals (Hammond-Kosack and Jones, 1996). Salicylic acid (SA) and hydrogen peroxide (H_2O_2) are two of the signals that play a crucial role in the activation and regulation of HR-related local and systemic defense responses (Delaney et al., 1994; Levine et al., 1994; Alvarez et al. 1998).

On the other hand, a growing number of plant defense forms have been described which differ from these in their symptomatic appearance or the biochemical processes accompanying them. One such frequently observed resistance phenomenon in plants is the "high sugar resistance" phenotype (Horsfall and Dimond, 1957), characterised by enhanced defense against pathogens, mediated through elevated levels of soluble carbohydrates and corresponding alterations in primary metabolism (Conrath et al., 2003). HR lesions are not visible in these plants, but the pathogen invasion is accompanied by significant H_2O_2 accumulation. Neither an increase in H_2O_2 content nor HR lesions can be observed in *Arabidopsis dnd1* mutants that possess characteristic resistance to avirulent *Pseudomonas syringae* (Clough et al., 2000). However, the assortment of biochemical responses of these "defense, no death" (*dnd*)-type plants includes a marked, prompt increase in salicylic acid levels. The fourth category of known defense mechanisms has proved to be $H_2O_2^-$ and SA-independent. While jasmonic acid (JA) and ethylene have been shown to be important for the induction of these alternative responses, H_2O_2 and SA do not seem to be involved in the signalling processes (Durner et al., 1997; Dong 1998).

In previous work, a novel defense reaction was described, regulated by the *gds* gene in pepper and providing effective resistance against a wide range of pathogens without showing HR-like lesions or other necrotic symptoms (Szarka and Csilléry, 1995). Instead, local cell division, cell wall thickening and the disappearance of intercellular spaces can be observed in infected tissues of plants possessing the *gds* gene.

The long-term aim of the biochemical examinations was to provide experimental evidence that the general defense system regulates an as yet unknown defense process. As the first step in investigation, the H_2O_2 and SA metabolism was monitored in two different pepper lines inoculated with the *X. vesicatoria* bacterium. One of the pepper lines contained the *Bs-2* specific resistance gene, encoding the specific defense reaction. When breeding pepper for resistance against the *X. vesicatoria* bacterium, the symptomatic manifestation of this gene is taken to be the drying of the tissue and reddish discoloration. This response was compared with that exhibited by a pepper line containing the *gds* gene regulating the general defense reaction.

The spectrophotometric determination of H_2O_2 contents during the first ten hours of infection with the *X. vesicatoria* bacterium showed that there was no H_2O_2 burst in the leaves of plants possessing the *gds* gene, while there was a 4-fold increase in plants with the *Bs-2* gene, and this started at a very early stage of pathogen attack (30 min after inoculation) (Fig. 7). After the initial burst, the H_2O_2 level remained significantly higher for 8 h in the pepper line carrying the *Bs-2* gene, compared with the non-infected control samples and the plants with the *gds* gene, after which it returned to the control level. At the same time, the amount of H_2O_2 remained constant in plants containing the *gds* gene, showing no change compared to the non-infected control plants within the timeframe of the measurements. The simultaneous determination of SA levels using the same plant material revealed minimal overlap between the regulation of the general defense system and the SA-dependent form of resistance. While the accumulation of SA was detected in the pepper line with the *Bs-2* gene during the first 10 h after infection (0.01–1.2 μg g^{-1} FW), the SA level was below the HPLC detection limit in plants with the *gds* gene in the course of repeated analyses (data not shown).

On the basis of the results, it appears that, unlike plants displaying rapid tissue destruction or 'high sugar resistance', plants possessing the *gds* gene do not require a H_2O_2-mediated oxidative burst for defense, and the regulation of the general defense system takes place through SA-independent pathways. The question now is whether jasmonic acid and ethylene take the place of SA and H_2O_2 in the control of the general defense system or whether it has an unknown regulatory process that remains to be elucidated.

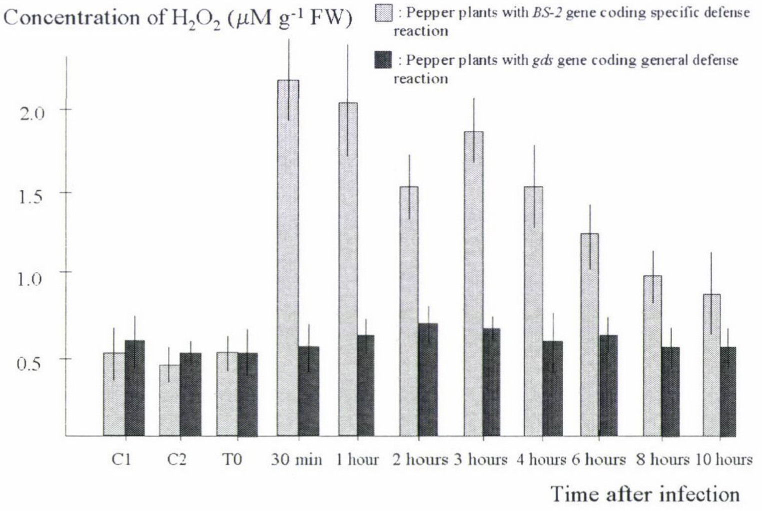

Fig. 7. Spectrophotometric determination of H_2O_2 contents in pepper leaves infected with *Xanthomonas vesicatoria* bacterium during the first 10 h after infection. Fully expanded leaves of pepper lines possessing either the *Bs-2* specific resistance gene or the *gds* gene were ground in liquid nitrogen (0.5 g tissue per sample) and homogenised in perchloric acid. The homogenates were neutralised and applied to Dowex 1X8 - 100-200 columns. H_2O_2 was eluted from the column and was determined by a spectrophotometric assay as described by Dorey et al. (1998). H_2O_2 was calculated by measuring a gradient of its known concentrations. The significance of differences between treatment groups was determined using Student's *t*-test followed by Fisher's LSD test as appropriate. If differences were considered significant for $P < 0.05$, means were separated by LSD at $P = 0.05$. Results for continuous variables are expressed as means ± SD. Abbreviations: C1 = non-infected control, C2 = leaves inoculated with sterile deionised water, T0 = samples were harvested immediately after *Xanthomonas* inoculation.

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References

Alvarez, M. E., Pennell, R. I., Meijer, P., Ishikawa, A., Dixon, R. A., Lamb, C. (1998): Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. *Cell*, **92**, 773–784.

Clough, J. S., Fengler, K. A., Yu, I., Lippok, B., Smith, R. K., Bent, A. F. (2000): The *Arabidopsis dnd1* "defense, no death" gene encodes a mutated cyclic nucleotide-gated ion channel. *Proc. Natl. Acad. Sci. USA*, **94**, 4800–4805.

Conrath, U., Linke, C., Jeblick, W., Geigenberger, P., Quick, W. P., Neuhaus, H. E. (2003): Enhanced resistance to *Phytophthora infestans* and *Alternaria solani* in leaves and tubers, respectively, of potato plants with decreased activity of the plastidic ATP/ADP transporter. *Planta*, **19**, 75–83.

Csilléry, G., Szarka, E., Sárdi, É., Mitykó, J., Kapitány, J., Nagy, B., Szarka, J. (2004): The unity of plant defense. Genetics, breeding and physiology. *Proceedings of the XIIth Eucarpia Meeting on Genetics and Breeding of Capsicum and Eggplant*. Noordwijkerhout, The Netherlands. pp. 147–153.

Delaney, T. P., Ukness, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, N., Gaffney, T., Gut-Rella, M., Kessmann, H., Ward, E., Ryals, J. (1994): A central role of salicylic acid in plant disease resistance. *Science*, **266**, 1247–1250.

Dong, X. (1998): SA, JA, ethylene and disease resistance in plants. *Current Opinion in Plant Biology*, **1**, 316–323.

Dorey, S., Baillieul, F., Saindrenan, P., Friting, B., Kauffmann, S. (1997): Spatial and temporal induction of cell death, defense genes, and accumulation of salicylic acid in tobacco leaves reacting hypersensitively to a fungal glycoprotein elicitor. *Mol. Plant Microbe Interact.*, **10**, 646–655.

Dorey, S., Baillieul, F., Saindrenan, P., Friting, B., Kauffmann, S. (1998): Tobacco class I and II catalases are differentially expressed during elicitor-induced hypersensitive cell death and localised acquired resistance. *Mol. Plant Microbe Interact.*, **11**, 1102–1109.

Durner, J., Shah, J., Klessig, D. F. (1997): Salicylic acid and disease resistance in plants. *Trends in Plant Science*, **2**, 266–274.

Flor, H. H. (1955): Host-parasite interactions in flax rust – Its genetics and other implications. *Phytopathology*, **45**, 680–685.

Hammond-Kosack, K. E., Jones, J. D. G. (1996): Resistance gene-dependent plant defense responses. *Plant Cell*, **8**, 1773–1791.

Horsfall, J. G., Dimond, A. E. (1957): Interactions of tissue sugar, growth substances, and disease susceptibility. *Z. Pflanzenkr. Pflanzenschutz*, **27**, 415–421.

Klement, Z., Farkas, G., Lovrekovich, L. (1964): Hypersensitive reaction induced by phytopathogenic bacteria in the tobacco leaf. *Phytopathology*, **54**, 474–477.

Levine, A., Tenhaken, R., Dixon, R., Lamb, C. (1994): H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell*, **79**, 583–593.

Ryals, J. A., Neunschwander, U. H., Willits, M. G., Molina, A., Steiner, H. Y., Hunt, M. D. (1996): Systemic acquired resistance. *Plant Cell*, **8**, 1809–1819.

Szarka, J., Csilléry, G. (1995): Defence systems against *Xanthomonas campestris* pv. *vesicatoria* in pepper. *Proceedings of the IXth Eucarpia Meeting on Genetics and Breeding on Capsicum and Eggplant*. Budapest, Hungary. pp. 184–187.

Szarka, J., Csilléry, G. (2001a): General defense system in the plant kingdom. *Int. Jour. of Hort. Sci.*, **7**(1), 79–84.

Szarka, J., Csilléry, G. (2001b): General defense system in the plant kingdom II. *Int. Jour. of Hort. Sci.*, **7**(3–4), 73–77.

Szarka, J., Sárdi, É., Szarka, E., Csilléry, G. (2002): General defense system in the plant kingdom III. *Int. Jour. of Hort. Sci.*, **8**(3–4), 45–54.

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Review

GENE FUNCTIONING IN PEPPER

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The biosynthetic pathways of essential and secondary products are well known in most plants. The rapid development of molecular genetic techniques in recent decades has facilitated the isolation and functional characterisation of genes from various organisms. Although pepper is not an optimal model for genomic analyses, many important genes have been isolated and characterised from this plant. In the present paper, pepper genes with known functions, such as taste or colour formation or the development of resistance responses, are reviewed, together with other information on pepper genomics. Flowcharts demonstrating the biosynthetic pathways described in the paper can be found in various places, e.g. on the Internet.

Key words: pepper, *Capsicum annuum,* gene function, gene expression, stress resistance

Introduction

Pepper is one of the most widespread vegetable or spice crops in the world. In contrast with many other crops, however, the possibility of isolating pepper genes is at present extremely limited. One of the main reasons for this is the enormous size of the pepper genome, which is 3–4 times as big as that of tomato and twenty times that of *Arabidopsis thaliana*, while it also has an extremely high proportion of repeated sequences. One possible technique for gene isolation is cloning based on the information from molecular genetic maps with high resolution. Another method is gene isolation based on induced gene expression. Using this technique candidate genes whose expression is linked to the given trait can be selected through the differential comparison of cDNA clone libraries synthesised from mRNAs. Further analysis is then carried out to identify the desired gene. With this method information is only obtained about the coding sequence of the given gene.

Using map-based or positional cloning, the bacterial spot resistance gene, *Bs2*, was isolated from pepper, while numerous genes, primarily for stress resistance, have been isolated from cDNA clone libraries. In some cases the functioning of these genes at the molecular level is known, while for others an informed guess can be made.

This review provides information on genes isolated from pepper, a knowledge of which opens up new vistas for pepper breeding in general, and in particular for breeding for single genes or gene products. In the descriptions the emphasis is placed on the importance and functioning of the gene.

Genes for flavour and pigments

Fruit ripening is a complex, genetically programmed process requiring the simultaneous expression of numerous genes and involving increased respiration rate and ethylene production, while the metabolism shifts towards the production of carbohydrates, organic acids, proteins and secondary metabolic products. In pepper, as in many other fruits, the chloroplasts become differentiated into non-photosynthesising chromoplasts, leading to the degradation of chlorophyll and starch and to extensive structural rearrangements.

Pungency and non-pungency

The burning sensation felt when eating pungent pepper is caused by the capsaicinoid alkaloids produced exclusively by members of the *Capsicum* genus. In capsaicinoids fatty acid side-chains with various degrees of saturation and length are attached to vanillylamine. The two most frequent capsaicinoids are capsaicin and dihydrocapsaicin, the first of which is always produced in far larger quantities. Other naturally occurring capsaicinoids are norhydrocapsaicin, homo-dihydrocapsaicin, homocapsaicin, norcapsaicin and nornorcapsaicin (Curry et al., 1999). Capsaicinoids are found not only in the fruit, but also in the vegetative plant parts, such as the leaves and stems. They are present in far larger quantities in fruits growing near the plant tip than they are in those on lower parts. However, the capsaicinoids in the vegetative organs exhibit different ratios to those in the fruit. It is interesting to note that dihydrocapsaicin is present in the largest quantity in the vegetative organs.

In classical genetic terms, pungency is a dominant trait controlled by a single major gene on the *C* locus, while mildness is recessive (Deshpande, 1935). The synthesis and accumulation of capsaicinoids takes place in the vacuoles in the epidermal cells of the placenta, after which they are secreted into the intercellular space of the cuticular and epidermis layers of the placenta, where they can be seen with the naked eye in the form of pale yellow or orange drops in the most pungent peppers (Ohta, 1963). Capsaicinoid production begins less than three weeks after fertilisation and generally continues throughout the fruit ripening process (Suzuki et al., 1980; Sukrasno and Yeoman, 1993).

Capsaicinoids are not produced in other organs of pepper; if the flower buds are removed from the plant these compounds cannot be detected in the vegetative organs.

Capsaicinoids are an intermediary product of the phenylpropanoid biosynthetic pathway and are synthesised via the condensation of vanillylamine and a short-chain fatty acid. Vanillylamine is produced via the phenylpropanoid biosynthetic pathway, while the fatty acid is formed from the amino acid valine (Fujiwake et al., 1982a, b; Ochoa-Alejo and Gomez-Peralta, 1993). Intermediary products of numerous other secondary metabolites (lignin, flavonoids, phytoalexins, pigments) are also formed in the initial steps of the phenylpropanoid biosynthetic pathway. Many of the genes coding for the enzymes involved in these initial steps have been cloned, including phenylalanine-ammonia liase (PAL) (Joos and Hahlbrock, 1992; Nagai et al., 1994), cinnamate-4-hydroxylase (Ca4H) (Fahrendorf and Dixon, 1993) and coffein *O*-methyltransferase (COMT) (Lee et al., 1998), the expression level of which is closely related to the pungency of the fruit. The intensity with which transcripts of these genes are formed is greatest in the most pungent tissue, the placenta, and is thus correlated with the pungency characteristic of the variety, being most intense in the most pungent variety, *C. chinense* cv. Habanero, and least in the placenta of mild varieties. *Pal, Ca4h* and *Comt* are minor gene families in pepper and exhibit over 85% homology with the corresponding genes in other plants. The *Pal* gene family includes four genes found in tobacco and tomato, which are closely related to pepper, and more than forty found in potato (Joos and Hahlbrock, 1992). Ca4h is a cytochrome P450-dependent monooxygenase, and as such could be the member of a gene superfamily (Chapple, 1998). *Comt* belongs to the *O*-methyltransferase superfamily and is a member of the *o*-diphenol-*O*-methyltransferase subgroup.

The expression of phenylpropanoid genes in the placenta tissue varies with the level of development and the pungency. For all three genes the transcription level is higher in the wall, placenta and seeds of immature fruit. Maximum accumulation is reached in the early stages of fruit development and the more pungent the fruit the greater the accumulation of transcripts. The relatively intense expression of *Pal* and *Ca4h* in immature seeds can be attributed to the role of these two enzymes in the extensive lignification associated with seed development. The expression of these two genes differs in the immature seeds of different pepper varieties, but the reason for this diversity is not yet known (Curry et al., 1999).

Not all the members of the capsaicinoid group cause a burning sensation. Capsiate, the non-pungent ester isoster of capsaicin, and its dihydro derivative are the major capsaicinoids of sweet peppers. The characteristic difference between the sensory properties of capsaicin and capsiate is due to the way the vanillyl and acyl moieties are linked, involving an amide bond in capsaicin and an ester bond in capsiate.

Capsaicin is known to cause apoptosis, or cell death, in tumour cells. Non-pungent capsiate has also been shown to cause apoptosis, which is preceded by the production of reactive oxygen species, resulting in a considerable reduction in the mitochondrial transmembrane potential. Nor-dihydrocapsiate in particular exhibits intense chemopreventive activity. Capsiates and their synthetic analogues probably exert this extremely positive physiological effect through the disturbance or inhibition of processes in the synthetic pathways involved in tumour formation and inflammation. In addition to their favourable dietary properties, various capsaicinoids are also important raw materials for pharmaceutical research and development (Macho et al., 2003).

Carotenoids

Plant carotenoids are essential isoprenoid pigments found in all green plant organs, being responsible for the red and yellow colours, and for intermediate shades, in fruit (Albrecht et al., 1995). They are bound to the chloroplasts as a complex formed with protein, and their oxygenated forms, the xanthophylls, are accessory pigments that play an important role in the transmission of light energy and the defence against photodestruction (Niyogi, 1999). The polyene chain of the carotenoids, which contains conjugated double bonds, is responsible for the characteristic colour and for the photochemical traits of the molecule (Hirschberg, 2001).

The carotenoids found in the chloroplasts play an indispensable role in photosynthesis, while those in the chromoplasts can be regarded as secondary metabolic products (Vishnevetsky et al., 1999). The carotenoids decisive for human health are the carotenes and vitamin A, or retinol, a molecule that provides a good example of the antioxidant activity of the carotenoids. All carotenoids containing a β-ring can be transformed into retinol and are thus the precursors of vitamin A (Hirschberg, 2001).

In higher plants the biosynthesis of carotenoids takes place in the plastids with the help of enzymes coded in the nuclear genome (Vishnevetsky et al., 1998). Like other isoprenoids, carotenoids are built from the compound isopentyl-diphosphate (IPP), which contains five carbon atoms. In the plastids IPP is formed from pyruvate and glyceraldehyde-3-phosphate via the 1-deoxyxylulose-5-phosphate (DOXP) synthetic pathway, the first enzyme of which is DOXP synthase (DXS). The DXS gene was isolated from pepper by Bouvier et al. (1998b), who named it CapTKT2, since it belongs to the transketolase group of enzymes.

IPP is isomerised to dimethylallyl diphosphate (DMADP) by IPP isomerase (*Ipi*). By the addition of three further IPP molecules, DMADP is converted into geranylgeranyl diphosphate (GGPP), a reaction catalysed by geranylgeranyl diphosphate synthase (GGPS). Several isoforms of GGPS are known, but it is not yet clear whether they are all involved in GGPP synthesis. What is certain is that phytoene is produced via the head-head condensation of two GGPP molecules, catalysed by phytoene synthase (*Psy*). Phytoene is then dehydrogenated into ζ-carotene or lycopene by phytoene desaturase (*Pds*) and ζ-carotene desaturase. The following step is the cyclisation of lycopene, which is an important branching point in the synthetic pathway, since one branch leads to the production of β-carotene and its derivatives, the xanthophylls (zeaxanthin, violaxanthin and neoxanthin), while the other possible synthetic pathway produces carotenoids containing a β-ring and an ε-ring, such as α-carotene and lutein (3,3-dihydroxy-α-carotene). Antheraxanthin and violaxanthin are formed with the help of epoxidase enzymes, so they are referred to as xanthophyll epoxides (Bouvier et al., 1996). When the xanthophyll epoxides are transformed into ketoxanthophylls, i.e. capsanthin and capsorubin, this causes the pepper fruit to turn red, while the chloroplasts are transformed into chromoplasts. The lutein synthesised on the other branch is the main xanthophyll in the leaves of higher plants. The concentration and composition of leaf xanthophylls in the chloroplasts are influenced by the light intensity, while the accumulation of specific carotenoids in the chromoplasts of the fruit and flower is regulated by the development process. In the course of fruit development, the *Ggps, Psy* and *Pds* genes in pepper chromoplasts are expressed most pronouncedly immediately prior to fruit colouring (Hirschberg, 2001).

Isopentyl diphosphate is the universal precursor of natural isoprenoids and is produced from mevalonic acid or from other non-mevalonic substrates. Two transketolases involved in the mevalonate synthetic pathway are known in pepper, CapTKT1 and CapTKT2. The genes coding for these two transketolases have distinct and important roles. CapTKT1 is involved in integrating the pentose phosphate and glycolytic cycles of the plastids, while CapTKT2 is responsible for the synthesis of isoprenoid in the plastid via the non-mevalonic acid synthetic pathway. CapTKT2 catalyses the formation of 1-deoxyxylulose-5-phosphate, the immediate precursor of IPP, from pyruvate and glyceraldehyde-3-phosphate. CapTKT1 is expressed constitutively almost throughout the process of chloroplast-chromoplast transformation, while CapTKT2 is overexpressed during this period, probably in order to provide the IPP required for the increased level of carotenoid biosynthesis. Deoxyxylulose phosphate is also needed in the plastids for the biosynthesis of isoprenoid, thiamine (vitamin B1) and pyridoxine (vitamin B6) (Bouvier et al., 1998b).

Fruit colour genes in pepper

In pepper, capsanthin-capsorubin synthase (CCS) converts antheraxanthin and violaxanthin into the red xanthophylls capsanthin and capsorubin. A deletion in the *Ccs* gene leads to the accumulation of violaxanthin, resulting in the manifestation of the recessive yellow colour in pepper fruits (*y* locus). The *Ccs* gene probably arose due to the duplication of the lycopene β-cyclase (*Lcy-b*) gene. In tomato this duplicated gene (*Cyc-b*) has regained its original catalytic function, while in pepper it has a similar biochemical nature, but has achieved new enzymatic activity in the course of evolution (Hirschberg, 2001).

The colour of pepper fruits is determined by eight different colours, ranging from white to red, associated with various combinations of three independent genes (Table 1), *Y, C1* and *C2*, described by Hurtado-Hernandez and Smith (1985).

Table 1
Genetic determination of the colour of pepper fruit

Fruit colour	Genotype		
Red	Y^+	$C1^+$	$C2^+$
Light red	Y^+	$c1$	$C2^+$
Orange	Y^+	$C1^+$	$c2$
Light orange	Y^+	$c1$	$c2$
Orange-yellow	y	$C1^+$	$C2^+$
Light orange-yellow	y	$C1^+$	$c2$
Yellow	y	$c1$	$C2^+$
White	y	$c1$	$c2$

This grouping makes classification rather complicated, however, since segregating populations from a cross involving parents with different fruit colours exhibit a continuous variation in fruit colour, making it difficult to distinguish the various shades. Thorup et al. (2000) thus suggested dividing the colour shades into four major groups, as follows:

red (from red to light red)
peach (salmon pink to peach)
orange (orange to orange-yellow)
cream (lemon yellow to white)

Up till now the following genes involved in the carotenoid synthetic pathway have been identified in pepper: geranylgeranyl diphosphate synthase (Ggpps), phytoene synthase (Psy), phytoene desaturase (Pds), ζ-carotene desaturase (Zds), zeatin epoxidase (Ze) and capsanthin-capsorubin synthase (Ccs). These genes can be detected using the specific primers listed in Table 2.

Table 2
Primer sequences used by Thorup et al. (2000) to detect the carotenoid genes of pepper

Gene	Gene bank No.	Fragment size, bp	Primer sequence (5'–3')
Ggpps	X80267	1.200	F GAACCTTGTTGATTTATGGGC R CCAACATAAGCACACTGAAAG
Psy	X68017	1.200	F TGGGCATCGCACCTGAATCAA R GTCCAGTATCCTGCGGTACAA
Pds	X68058	420	F TTCGACTTGTTTCTGCTGTCA R CATCCCTTGCCTCCAGCAGTA
Zds	X89897	400	F GCTCCAAAAGGGCTATTTCC R TCCCATTTCAATGTGGTTCC
Ze	X91491	1.900	F ATGGCATAAGGTCTAAGGTAC R CTCAGATAGTCTGCAATGTTG
Ccs	X77289	1.490	F CTAATGGAAACCCTTCTAAAGC R AATTCAAAGGCTCTCTATTGCT

F: forward; R: reverse

The red colour of pepper fruits is caused by the dominant Y^+ allele coding for red carotenoid pigments, while the recessive *y* allele is responsible for yellow colour. It was demonstrated by Lefebvre et al. (1998) that the capsanthin-capsorubin synthase (*Ccs*) gene is only activated in the final stages of fruit ripening and that the yellow phenotype is only possible due to a deletion in the *Ccs* gene. The *Y* gene in the classical sense is thus equivalent to the *Ccs* gene in the molecular sense. It was found by Popovsky and Paran (2000) that the deletion involved a 220 bp sequence at the 3' end of the *Ccs* gene, which is missing in the recessive allele. Plants with red or peach-coloured fruit contain the wild-type *Ccs* gene, while the mutant allele is to be found in plants with orange, yellow or white fruit. However, two different genotypes may result in orange fruit. These results are in agreement with the findings of Thorup et al. (2000), who also reported that red and peach-coloured fruits were determined by the Y^+ allele and orange and cream fruits by the *y* allele.

In addition to the *Ccs* gene, the *Psy* gene is also present in red and peach-coloured fruits, while it is missing from orange and cream-coloured plants. The difference between the red and peach, or the orange and cream groups can be attributed to different levels of the same pigment. This suggests that the *C2* gene, which has a substantial effect on the total accumulation of carotenoids, is identical with the *Psy* gene. The hypothetical function of the *Psy* gene in regulating carotenoid accumulation is confirmed by investigations on transgenic tomato. Tomato lines containing the antisense *Psy* gene exhibited a reduced carotenoid level in the fruits. It is thought that the difference between the *Psy* alleles may be in the cis-regulatory sequences or in the enzyme structure.

Based on the analysis of *Ccs* and *Psy* genes, the Hurtado-Hernandez and Smith pepper fruit colour model should be modified to include the fact that capsanthin and capsorubin production also takes place in the peach group (orange and light orange), though the quantities are only about a hundredth of those in red fruits. The *C1* gene may also influence the level of carotenoid production, but this gene has not yet been identified in the molecular sense. The accurate identification of the genes involved in colour manifestation is complicated by the fact that the same orange-coloured shades may be determined by different pigment combinations. Depending on the gene complement, orange-coloured lines may have the combinations $yC2^+$ or Y^+c2, so they may contain capsanthin and capsorubin, but these compounds may also be missing.

Stress resistance genes

Carotenoids in the defence against oxidative stress

The expression of carotenoid genes in pepper fruits is intensified by oxidative stress, thus accelerating carotenoid synthesis during the chromoplast differentiation process. This explains why the site of injury or microbial infection turns red on otherwise green fruit.

Numerous data indicate that reactive oxygen species (ROS) exert their effect at the molecular level. Due to the process of photosynthesis, which produces oxygen, the internal oxygen concentration is higher in plants than in any other type of living organism, leading to oxidative stress. Plants have developed various enzymatic and non-enzymatic defence mechanisms against oxidative stress effects. One non-enzymatic mechanism is the neutralisation of active oxygen species by isoprenoids such as carotenoids or tocopherol, while catalases, glutathion peroxidase, ascorbate peroxidase and superoxide dismutase are involved in the enzymatic neutralisation of ROS.

When pepper fruits ripen, large quantities of carotenoids, including the red pigments capsanthin and capsorubin, are formed as part of the chloroplast-chromoplast transformation process. The differentiation of chromoplasts in the pepper fruit begins in the ripe green stage and the complete process takes about two weeks.

As the result of injury or treatment with metflurazon herbicide or the inhibitors diflufenican or 2-(4-chlorophenylthio)-triethylamine the mRNA levels of the genes *Ggps, Psy, Pds* and *Lcy* exhibit a general increase. These inhibitors also cause an increase in the transcript level of xanthophyll-producing enzymes, zeaxanthin epoxidase (Zepd) and Ccs, suggesting that the blocking of carotenogenesis activates the genes expressed in carotenoid biosynthesis. There may be other reasons for the induction of these genes, apart from feedback regulation. One possibility is photooxidative stress due to the lack of biologically active carotenoids, which are capable of protecting the chlorophyll. Tests involving the *Ccs* gene indicated that the expression of the gene was controlled by reactive oxygen species. In summary, it can be said that oxidative stress plays a key role in the induction of carotenoid biosynthesis in the chromoplasts and in the chloroplast-chromoplast transformation (Bouvier et al., 1998a).

Modification of isoprenoid biosynthesis as a response to stress

Isoprenoids are mostly produced by plants and include the membrane sterols involved in cell growth and development, growth regulators (gibberellins, abscisic acids) and photosynthetic pigments (chlorophyll, carotenoids). They also include secondary metabolic products such as defence components (phytoalexins), pharmaceutically important compounds, flavour and odour compounds, natural rubbers and isoprenoids with special functions in plants.

Isoprenoid biosynthesis in plant cells may follow one of two pathways. One involves the biosynthesis of sesquiterpenes, triterpenes and polyterpenes, primarily in the cytosolic or endoplasmic reticulum, via the acetate/mevalonate synthetic pathway, and the other the biosynthesis of isoterpenes, monoterpenes, diterpenes, tetraterpenes and certain prenylated quinones in the plastids via the glyceraldehyde-3-phosphate-pyruvate pathway (Kellogg and Poulter, 2002).

Stress, or treatment with an elicitor, induces changes in the normal course of isoprenoid biosynthesis, leading to massive isoprenoid phytoalexin biosynthesis.

Intense ketocarotenoid synthesis takes place during the ripening of pepper fruits, a process that includes the production of capsanthin and capsorubin. This synthetic pathway can be diverted to induce intensive lycopene synthesis by treatment with CPTA [2-(4-chlorophenylthio)triethylamine hydrochloride]. As a response to stress, active capsidiol synthesis takes place in the cytoplasm.

Among the enzymes in the cytosol, the expression of farnesyl pyrophosphate is constitutive during the ripening of pepper fruits, while no farnesyl pyrophosphate cyclase, or 5-*epi*-aristolochene synthase, can be detected. The isoprenoid biosynthesis observed in the plastids is developmentally controlled, while in the cytoplasm it is partially controlled by environmental signals (Hugueney et al., 1996).

In plants, 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) catalyses the conversion of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) into mevalonic acid. The latter is an important compound in the biosynthesis of phytosterols, sesquiterpene phytoalexins and carotenoids. It is thought that HMGR may be one of the enzymes limiting the biosynthetic ratio of phytosterols of cytosolic acetate/mevalonate origin. The HMGR members coded by multigenic families respond differently to various external stimuli, including pathogen infection, and are expressed tissue-specifically and also as a function of the developmental phase.

Among the three different HMGR isogenes of pepper, *Hmg2* can be assumed to play a role in the defence system on the basis of its structure and the way it is expressed in response to *Phytophthora capsici* infection. *Hmg2* was isolated from cDNA clone libraries prepared from the roots of pepper infected with *P. capsici* using the cDNA of rice *Hmg2* as a hybridisation probe. The gene contains a 1815 bp open reading frame (ORF) coding for 604 amino acids. Using this pepper *Hmg2* gene as a probe, Ha et al. (2002) isolated genes *Hmg1* and *Hmg3* from a cDNA clone library prepared from the leaves, stem and flowers of healthy pepper plants.

The amino acid sequence of pepper HMGR2 exhibits the greatest homology with that of the HMGR2 enzyme of potato (89%), tomato (91%) and tobacco (89%), suggesting that the HMGR2 gene is highly conserved.

The *Hmg2* gene was induced within an hour of infection with *P. capsici* in pepper roots, and the number of transcripts increased continually for the next 48 hours. No transcription products for *Hmg1* were found in the roots. All three HMGR genes were expressed intensively during the early phases of fruit development. *Hmg1* and *Hmg2* transcripts gradually disappeared as the fruit developed, and could no longer be detected in ripening fruit. By contrast, *Hmg3* was strongly expressed during both fruit development and ripening.

After infection, the regulation of *Hmg2* and of the sesquiterpene cyclase and farnesyl pyrophosphate synthase genes required for the biosynthesis of the sesquiterpene phytoalexins involved in the defence system is well coordinated, taking place in a predetermined order.

In plants, HMGRs primarily play an important regulatory role in isoprenoid biosynthesis, as indicated by their expression during fruit development. In addition, the structure of HMGR2 is close to that of defence HMGR isoforms, and its nucleotide sequence exhibits great homology with that of plant defence HMGRs. The rapid expression in response to *P. capsici* infection suggests that it is part of the inducible defence mechanism and could be an important component in the biosynthesis of phytoalexins.

Catalases in the defence against oxidative stress

Catalase is a tetrameric haem-containing enzyme found in all aerobic organisms, which plays a key role in protecting the cell against oxidative stress by converting hydrogen peroxide (H_2O_2) into oxygen and water. During metabolism there is always a danger that the oxygen level may decline temporarily, allowing toxic reactive oxygen species (ROS) to form. This accumulation of toxic ROS, which may occur during normal metabolism or as the result of environmental stress, can cause severe cell damage. In order to minimise the oxidative stress caused by ROS, aerobic organisms have developed a wide range of enzymatic and non-enzymatic defence mechanisms. One component of enzymatic defence is the catalase gene family, in which the various genes code for various proteins that are differently localised and regulated within the cell (Kwon and An, 2001).

The peroxisomal catalase isolated from pungent pepper, CaCat1 (*Capsicum annuum* Catalase 1) belongs to the first of the three classes of catalase and exhibits 99% homology with the derived amino acid sequence of the catalase gene isolated from green pepper and 95% homology with the amino acid sequence of the NtCat1 (*Nicotiana tabacum* Cat 1) gene, which is also of *Solanaceae* origin. The level of homology is only 68–78% with the NpCat1 gene (*Nicotiana plumbaginifolia* Cat1) and less than 50% with humans, yeast and the *Escherichia coli* bacterium.

CaCat1 mRNAs are found in the largest quantity in the stems of pepper plants, while there are fewer in the leaves and only traces in the roots. The high level of catalase activity in the stems suggests that the intense respiration associated with the transport of various metabolic products, water and minerals causes substantial oxidative stress in pepper stems.

Among the various developmental phases of the fruit, CaCat1 transcripts are most plentiful in the ovary, while only half as many are found in very young fruit, a third in developing fruit, a tenth in mature red fruit and only traces in mature green fruit. Interestingly enough, other antioxidant enzymes, such as Cu/Zn superoxide dismutase, are most abundant in mature red fruit and ascorbic peroxidase in very young green fruit, while the level of Mn superoxide dismutase changes very little in the course of fruit development. It seems likely that CaCat1 provides defence against oxidative stress in the early stages of fruit development, while superoxide dismutase and ascorbic peroxidase start to function under the changed conditions found in the later phases of development (increases in cell size and sugar content, hormone effects, pigment changes).

In response to the abiotic stress caused by aluminium or common salt the CaCat1 gene is induced in the roots, where it can hardly be detected under normal conditions. This can be attributed to the oxidative stress caused by the fatty acid peroxidation effect of aluminium and the enhanced superoxide-generating effect of salt. The activity of the CaCat1 gene exhibits rhythmic changes with a transcription peak at the end of the dark period and the beginning of the light period, i.e. around dawn. In this respect its activity is similar to that of the NpCat1 gene, but differs from that of the NtCat1 gene, the expression of which exhibits no rhythmic changes in response to dark/light.

As can be seen from the above, the regulation of the CaCat1 gene is rather complex in pepper, including both external environmental signals, such as light, and internal signals related to the stage of development (Kwon and An, 2001).

Peroxidases

Various peroxidases play an important role in plant development and defence mechanisms. The peroxidases include a large number of isoenzymes with various functions, and are involved in the deposition of cell wall-strengthening materials such as lignin, suberin and extensin. One of the most important peroxidases is ascorbate peroxidase, an antioxidant that removes the H_2O_2 accumulating as the result of injury, pathogen attack or environmental stress. When the pathogen penetrates the plant, ascorbate peroxidase activity is inhibited by salicylic acid in the course of the hypersensitive response, leading to hydrogen peroxide accumulation and to the acceleration of programmed cell death.

Do et al. (2003) isolated three cDNA clones that were induced by infection with the Bv5-4a avirulent strain of *Xanthomonas campestris* pv. *vesicatoria* in pepper. These genes, *CAPOA1, CAPOT1* and *CAPO1* are induced even by an extremely low level (10 μM) of exogenous hydrogen peroxide treatment. The *CAPOA1* and *CAPOT1* genes are expressed very quickly, within an hour of treatment, and are involved in the detoxification of the excess hydrogen peroxide, while *CAPO1* is activated later, some 12 hours after treatment, and probably acts as an indicator molecule.

Biotic stress resistance

As the result of the joint evolution of hosts and pathogens, plants have developed refined mechanisms with which to protect themselves against the damaging effects of pathogens. In addition to physical and chemical obstacles to infection, there is a wide range of mechanisms that come into effect after pathogen attack (Kombrink and Somssich, 1995). If these defence mechanisms are induced sufficiently rapidly, in a well coordinated manner, the plant will be resistant to the given disease. In susceptible plants the defence response is far slower. The major difference between resistant and susceptible plants is in the timely recognition of the attacking microorganism and the induction of an effective defence mechanism (Yang et al., 1997). The avirulence (*avr*) genes of the pathogen play a decisive role in infection. An *avr* gene product induces a defensive response from the plant provided the plant possesses the corresponding *R* gene that is capable of recognising the pathogen. This type of interaction is referred to as gene-for-gene resistance. The products of the *R* gene are receptors that recognise and bind the protein products of the *avr* genes, thus initiating a signalling chain reaction that activates the defence mechanism and eventually leads to the destruction of the infected cell. This is an extremely specific relationship, so if the *avr* genes change or are inactivated due to a mutation, the pathogen may go unrecognised. Defence mechanisms dependent on *R* genes are often associated with a hypersensitive response (HR) or with the destruction of cells penetrated by the pathogen (Jones, 1996). A wide range of distantly related *R* genes capable of recognising different pathogens may be found within a single plant species.

In recent years numerous resistance genes have been cloned. These can be classified in various groups on the basis of certain traits, particularly their probable protein structure. For the majority of resistance traits as many as five or more genes may be linked with each locus. The wide variety of *R* genes has been generated by gene duplications followed by point mutations or deletions, or by the duplication of or reduction in the number of DNA repetitions coding for leucine-rich components within the gene. An analysis of the gene families indicates that the development of a given resistance gene is followed by recombination, duplication or transposition, while not all the members of the gene family make a functional contribution to the development of resistance (Song et al., 1997). This specific genetic system was evolved by living organisms to maintain diversity, thus ensuring their survival in a changing environment. Extremely polymorphic resistance genes are found in most plant species, though their sequences also exhibit a great deal of similarity. It has long been assumed that changes at the DNA level play a key role in the evolution of resistance genes, since this makes them capable of responding to genetic changes in the pathogens (Pryor and Ellis, 1993; Ellis et al., 2000). This hypothesis is confirmed by the observation that recombination in a resistance locus is associated with the manifestation of a new type of resistance phenotype (Richter et al., 1995). The rearrangement of recombining sequences may take place in two ways: there may be balanced exchanges, where the genes of the complex only recombine with the corresponding genes of a homologous complex, and unbalanced exchanges, where each gene may recombine with any gene in the homologous complex. Nevertheless, there is far less likelihood that recombination will take place between widely differing sequences that between similar genes at a complex locus. The proportion of chimeric alleles arising due to balanced and unbalanced rearrangements is determined not only by the number of sequence rearrangement events, but also by the concomitant selection that encourages the evolution of new types of resistance traits. The selection pressure exerted by the pathogens on factors involved in the development of resistance also contributes to maintaining wide variety among the *R* genes. A comparison of the sequences of resistance genes revealed that, due to gene conversion, sequence rearrangements also take place regularly between resistance genes that are not genetically linked (Bendahmane et al., 1999). Apart from their great sequence homology, the resistance genes are also characterised by the fact that, regardless of their function, they are often found in closely linked clusters on certain chromosome segments. Comparative mapping of various plant genera has revealed close linkage between genes for resistance to taxonomically diverse pathogens. *R* genes make up a substantial portion of the plant genome. Genome sequencing data have shown that the nucleotide-binding site–leucine-rich repeat (NBS-LRR) class of *R* genes makes up 1% of the *Arabidopsis thaliana* genome.

Plants respond to pathogen attack or external environmental stress by producing a range of pathogenesis-related (PR) proteins. The induction and accumulation of PR proteins can often be observed in infected tissues in the case of incompatible plant–pathogen interactions. Though this is not only true of resistant plants, the time and magnitude of induction differs in resistant and susceptible lines, and there are also differences in the protein isoforms produced during these processes.

Among the PR proteins, the chitinases and the β-1,3-glucanases are known to be connected with defence against fungal infection.

Chitinases

In plants chitinases form one of the most important enzyme groups involved in mechanisms related to pathogenesis, and their quantities rise enormously in tissues in response to injury or to infection with fungi, bacteria or viruses. The majority of them belong to the major PR genes and catalyse the hydrolysis of chitin (a linear homopolymer constructed from β-1,4-linked *N*-acetyl-D-glucosamine residues). In plants, chitinases play a role in defence against pathogens and abiotic stress. Their expression can be induced by pathogenic infection, ethylene or salicylic acid treatment, injury, osmotic stress, mercury chloride, ozone and UV radiation. In addition they may also be expressed in connection with plant morphogenesis and development. It is worth noting that plants produce chitin despite the fact that they possess no cell organelles that contain chitin. In farm practice chitinases are applied for the effective control of phytopathogenic fungi and insects.

Chitinases can be divided into five classes, I–V, on the basis of their amino acid sequence. *CAChi2*, from class II, and *Chi3-P1*, from class III, have been isolated from pepper. The amino acid sequence of class II chitinases is very similar to that of class I except that a cysteine-rich domain is missing from the N terminal and a signal peptide from the C terminal. Class II chitinases are secreted into the apoplasts. The *CAChi2* gene is constitutively expressed in pepper roots and flowers, while it is only induced in leaf and stem tissues and in the fruit in response to virulent or avirulent pathogen infection. The rapid, intense chitinase activity observed in the course of the incompatible interaction probably plays an important role in the response of pepper plants to bacterial and oomycetic pathogens (Hong et al., 2000). It can thus be concluded that the expression of the *CAChi2* gene is organ-specific and that *CAChi2* chitinase could be a permanently functioning part of the preventive defence system in these organs. It probably serves to inhibit the formation of colonies by soil-borne fungi in the roots. Certain soil-borne pathogens are capable of penetrating the root-tip meristem, epidermis and cortex, but not the root epidermis or vascular tissues, where chitinase genes are constitutively expressed and form a biochemical barrier to avirulent pathogens.

Some PR genes are also expressed in healthy reproductive tissues; for instance, β-1,3-glucanases, thionins and *CAChi2* are also active in healthy pepper flowers. It is unlikely, however, that the chitinases have a protective function in flowers; the accumulation of *CAChi2* mRNAs in the central part of the placenta and in the internal integuments of the ovule seems to suggest a role in flower differentiation (Hong and Hwang, 2002).

The sequences of class III proteins do not resemble those of chitinases in other classes, exhibiting greater similarity with those of fungal enzymes involved in morphogenesis, which are typically extracellular proteins with lysosome activity. The *Chi3-P1* gene isolated from pepper by means of sequence-based PCR typing using degenerated primers was mapped to the P3 pepper chromosome. This gene codes for a 32-kDa chitinase consisting of 295 amino acid residues. It exhibits 62% homology with the P29060 clone of tobacco at the DNA level and 57% at the amino acid level. Phylogenetic analysis revealed a close relationship with a subgroup of the class III chitinases of *Arabidopsis thaliana*. The comparative analysis of a number of pepper inbred lines revealed single nucleotide polymorphism and point mutations. The clarification of the role played by the *Chi3-P1* gene in disease resistance will require the isolation of further members of the family in order to determine their functions (Cheng et al., 2002).

β-1,3-glucanase

The β-1,3-glucanases hydrolyse β-1,3-bonded glucanes, the major cell-wall components of oomycetes, and inhibit fungal growth, functioning synergically with chitinase. At the same time, this enzyme is able to liberate glucan fragments functioning as signal molecules from plant or fungus cell-walls, which then activate various defence systems in the plant. In addition to pathogen attack, β-1,3-glucanases can also be induced by abiotic elicitors such as ethylene, salicylic acid or methyl jasmonate. The β-1,3-glucanases are also involved in various physiological and development processes such as microsporogenesis, seed germination, and pollen and fruit development.

Phytophthora capsici is one of the most frequent soil-borne fungal pathogens of pepper. Infection of pepper stems with *P. capsici* induces a quantitative hypersensitive reaction. In resistant plants the necrotic lesions are smaller and fungal growth is inhibited, while in susceptible plants the necrotic symptoms spread over larger stem areas and the pathogen remains active, finally leading to the death of the plant.

In investigations carried out by Egea et al. (1999) the activity of β-1,3-glucanases was found to increase continuously in resistant plants after infection, reaching a maximum on the sixth day. By contrast, glucanase activity could not be detected in the intercellular fraction of the susceptible variety Yolo Wonder until the ninth day after infection.

In response to infection the activation of three isoenzymes could be detected in resistant plants, compared to two in Yolo Wonder. Intracellular glucanases were mainly found in the vacuoles and may be involved not so much in resistance as in non-specific defence mechanisms against *P. capsici*.

The rate at which β-1,3-glucanase is accumulated probably has a decisive role in the development of resistance. Hyphae penetrating between the cells are directly exposed to extracellular glucanases, the quantity of which is substantially higher in resistant plants than in susceptible ones. In addition, in resistant plants compounds are produced in the course of cell-wall hydrolysis that induce the production of capsidiol phytoalexin, which inhibits hyphal growth. In pepper, *P. capsici* resistance depends on the activity of β-1,3-glucanase and on the rate of synthesis, which leads to an unfavourable environment for fungal growth in the plant, thus allowing the plant to elicit a suitable response to the pathogen.

Various isoforms of β-1,3-glucanase occur in plants. Acidic and basic β-1,3-glucanases were isolated from pepper in response to *P. capsici* infection (Kim and Hwang, 1994). These genes are also induced by *X. campestris* pv. *vesicatoria* infection. Recently the *CABGLU* (*C. annuum* β-1,3-glucanase) gene has been isolated from a cDNA clone library prepared from pepper leaves infected with the avirulent Bv5-4a strain of *X. campestris* pv. *vesicatoria* (*Xcv*) (Jung and Hwang, 2000). Based on the amino acid sequence derived from cDNA, the coding region exhibits a high degree of conservation with related *Solanaceae*: tobacco, potato and tomato. After infection with the virulent *Xcv* strain Ds-1, lesions did not appear even after 30 hours, while the symptoms of the hypersensitive reaction were visible on leaves inoculated with the avirulent strain Bv5-4a after a period of 18 hours. Irrespective of the incompatible or compatible interaction, *CABGLU* mRNAs accumulated intensively as the result of *Xcv* infection. In pepper stems there was a considerable reduction in *CABGLU* mRNA accumulation 48–96 hours after inoculation with the oomycete *Phytophthora capsici*. This substantial decline in transcription in later stages of infection may be due to the severe damage suffered by the infected stems. Inoculation tests suggest that β-1,3-glucanase only transmits part of the defence responses induced by pathogen infection.

Among the abiotic elicitors, etephon and methyl jasmonate (MeJa) have been found to induce the accumulation of β-1,3-glucanase mRNA. By contrast, salicylic acid, BABA (DL-β-2-amino-*n*-butyric acid) and benzothiadiazol do not activate the β-1,3-glucanase gene in pepper leaves. This indicates that ethylene and methyl jasmonate may be signal molecules for the activation of the β-1,3-glucanase gene, but that this activation is independent of salicylic acid.

The accumulation of *CABGLU* mRNAs is most intensive in pepper roots, suggesting that β-1,3-glucanase plays an important role in the preventive defence of sensitive tissues against pathogen infection. Weak expression can also be observed in the flowers, which could be due to the hydrolase involved in the formation of reproductive organs.

Capsidiol

The joint evolution of plants and microbes through a process of interaction has resulted in the exchange of various signals and responses. A pathogen attempting to colonise a host organism can expect to meet with a vigorous, rapid defence response that will inhibit further infection. These metabolic responses include the activation of the phenylpropanoid metabolism, the synthesis and accumulation of phytoalexins and the synthesis of PR proteins (Garcia-Pineda and Lozoya-Gloria, 1999). The main phytoalexin deployed by pepper against phytopathogenic microorganisms is the bicyclic sesquiterpene, capsidiol. Capsidiol accumulates in necrotic stem parts, where it inhibits fungal growth and exerts a fungitoxic effect. Gas chromatographic measurements made by Egea et al. (1996) demonstrated that the inhibition of fungal growth was perceptible in the stems of resistant pepper plants from a capsidiol concentration of 3.75 mM, and the fungitoxic effect from a concentration of 5 mM. The former concentration was achieved by the plants after six days. The decisive role of capsidiol in the development of *P. capsici* resistance is shown by the enhanced capsidiol accumulation in varieties with greater resistance.

The resistance response can also be induced in pepper cell suspensions using lyophilised mycelia or fungus filtrate, when maximum capsidiol production is reached after 18 or 12 hours, respectively. In addition to capsidiol, PR proteins with glucanase and chitinase activity are also produced. A clearly perceptible difference in the production of these compounds could be observed between cell suspensions of resistant and susceptible pepper varieties six hours after treatment. In the resistant line capsidiol was produced more rapidly than it could be transported to the intercellular space. Various kinds of hydrolase activity could be observed in both resistant and susceptible lines, but these were probably part of the general defence mechanism against the fungus. Nevertheless, certain glucanases and extracellular chitinases were only found in the resistant suspension. It thus appears that *P. capsici* signal molecules induce a multicomponent, dynamic system in pepper cell suspensions, in which the various defence mechanisms complement each other (García-Pérez et al., 1998).

5-epi-*aristolochene synthase*

One of the key enzymes in capsidiol biosynthesis is 5-*epi*-aristolochene synthase, which is coded by the PEAS (pepper 5-*epi*-aristolochene) gene (Bohlmann et al., 2002).

Like other members of the *Solanaceae*, such as tobacco, pepper responds to infection or stress treatment by producing sesquiterpene phytoalexins. An important step in this process is the utilisation of farnesyl pyrophosphate (FPP), a strictly regulated process, since FPP acts as a precursor for various compounds in extremely varied biosynthetic pathways. The squalene required for sterol biosynthesis is synthesised from two FPP molecules, while geranylgeranyl pyrophosphate (GGPP) arises from the addition of isopentyl pyrophosphate to an FPP molecule. GGPP is required for the synthesis of terpenes, e.g. gibberellins. The cyclic structures participating in sesquiterpene production also include an FPP molecule. The well-regulated cyclisation of FPP is essential if the required structure is to be produced. The enzymes responsible for these processes are sesquiterpene cyclases, and specific cyclases are required for the synthesis of various cyclic structures. Some sesquiterpene cyclases can be generated by various stimuli. In pepper and tobacco FPP is converted into 5-*epi*-aristolochene, which later takes part in the production of the bicyclic sesquiterpene phytoalexin capsidiol by means of hydroxylation.

Several sesquiterpene cyclase genes have been isolated from tobacco, so it is clear that sesquiterpene cyclase gene families exist, the members of which are regulated differently in the course of development or as a response to environmental stimuli. The 5-*epi*-aristolochene synthase (EAS) enzyme catalyses a key step in the synthesis of capsidiol, the most important sesquiterpene phytoalexin found in pepper and tobacco.

In the stems of pepper plants artificially inoculated with *Phytophthora capsici* a high level of transcription is observed 24 hours after infection for the mRNA of PEAS1 (pepper 5-*epi*-aristolochene synthase 1) and PEAS18, which differs from it at the nucleotide level. Gene expression is most intense in the stems, while it can still be detected in the roots but not in the leaves. This is in agreement with the fact that *P. capsici* infection induces a hypersensitive reaction in the stems and roots of pepper, while the leaves remain symptom-free. The pathogen-induced PEAS1 and PEAS18, and PEAS55, which exhibits great homology with PEAS1, appear to form a gene family consisting of 6–8 members. PEAS1 and PEAS55 probably arose from the same gene, g*PEAS*1. The exon/intron structure of the isolated g*PEAS*1 gene is very similar to that of the *TEAS4* (tobacco 5-*epi*-aristolochene synthase 4) gene isolated from tobacco, but the sequences found after the start codon are very different. The activity of the g*PEAS*1 gene can be induced by cellulase treatment that decomposes the plant cell-wall. The local expression of the gene in response to cellulase treatment is considerably faster than after infection with *P. capsici*: a substantial quantity of mRNA can be detected as early as two hours after treatment, with a maximum six hours after infection. It could be that the g*PEAS*1 gene induction arises as a local defence response, but the possibility that a systemic response of low intensity may appear later cannot be excluded (Zavala-Páramo et al., 2000).

Induction of defence responses using elicitors

The investigation of induction mechanisms for defence responses in plants has been greatly promoted by the use of isolated molecules of plant and microbial origin, known as elicitors, that are capable of inducing the same defence responses as true infection. Induced defence mechanisms only begin to function in plants when the pathogen is recognised, so the defence products are absent from healthy tissues or present in negligible quantities, and can only be detected when resistance is expressed. Consequently, these mechanisms represent an excellent model for the examination of cell signals, the monitoring of metabolic processes and gene expression, and the evaluation of specific elements involved in mechanisms leading to resistance.

In the majority of plant–pathogen interactions a number of induction mechanisms are expressed simultaneously. It is also known that certain elicitors may stimulate more than one defence response. The biosynthesis of phytoalexins is an inducible defence mechanism and also one of the main defence systems in higher plants. The sesquiterpene phytoalexins isolated from members of the *Solanaceae* family are closely correlated with the defence response given by the plant to pathogen invasion. Known elicitors of sesquiterpene phytoalexin production are cellulase originating from *Trichoderma viride*, and arachidonic acid (AA), a fatty acid isolated from *P. infestans* and not found in plants. In pepper fruit, seedlings and cell cultures, AA induces the accumulation of phytoalexin.

In pepper seedlings the stress caused by AA induces the simultaneous functioning of a number of genes. In addition to genes activated in the phytoalexin biosynthetic pathway, such as PEAS, genes involved in ethylene production are also expressed. Maximum ethylene production is observed after the transcription of the CA-ACCO [*C. annuum* 1-aminocyclopropan-1-carboxylate (ACC) oxidase] gene in both AA-treated and untreated fruits. The role of ACC synthase appears to be extremely important in ethylene production in the fruit. ACC oxidase is a multigene family, which could explain why, although the ethylene production occurs at the same time, differences are observed in the ACC transcript accumulation, since the ACC oxidase genes are expressed at different times. It is still not clear what role is played by the ethylene produced after AA treatment. As the ACC oxidase gene is expressed at various levels in pepper leaves, it is possible that it is related to aging (Garcia-Pineda and Lozoya-Gloria, 1999).

Ethylene is also involved as a signal molecule in the development of systemic acquired resistance. After pathogen infection, cells undergoing necrosis produce ethylene, which then induces the accumulation of defence-related proteins. Ethylene biosynthesis is also influenced by jasmonate (JA) and its methyl ester, compounds naturally occurring in plants and capable of inducing defence-related proteins.

Abscisic acid (ABA), which is also capable of inducing stress responses, has a function in changes in plant growth and development, particularly during seed formation and as a response to environmental stress factors such as water deficiency (Shinozaki and Yamaguchi-Shinozaki, 1997). Treatment with ABA makes plants more resistant to environmental stress and promotes post-stress recovery (Chandler and Robertson, 1994).

Systemic acquired resistance – the SAR8.2 small gene family

Plants are continually exposed to pathogen attack and their only means of defence is to activate resistance (Collinge and Slusarenko, 1987). Defence mechanisms may be of two types, depending on whether they are activated prior to infection or only in response to signals induced by the pathogen. The latter may provide protection not only at the site of infection, but systemically, in the whole plant. Local defence occurs after the specific recognition of the pathogen and takes the form of programmed cell death, known as the hypersensitive response (HR), which thus inhibits the penetration of the pathogen from infected cells to neighbouring healthy tissues. The narrow zone surrounding the HR region is characterised by the intense stimulation of the defence response (Dorey et al., 1998).

In contrast, systemic acquired resistance (SAR) provides protection to the whole plant via signal molecules, irrespective of the site of infection (Ryals et al., 1996). One such signal molecule is salicylic acid, which occurs naturally in plants. Both the local and systemic accumulation of salicylic acid appears to be indispensable if the signalling system inducing the expression of SAR is to function (Malamy and Klessig, 1992). In addition to salicylic acid, other signal molecules such as ethylene, systemin and jasmonate also play an important role in SAR development, in the course of which PR proteins (Ward et al., 1991) and other defence-related proteins, thionins, defensins and SAR8.2, are also activated.

The salicylic acid level in plants is related to the resistance to the attacking pathogen; a systemic rise in the salicylic acid level is essential for SAR induction.

SAR8.2 is a small gene family that can be induced by any stimulus leading to resistance (Ward et al., 1991).

The CASAR8.2A, B and C (*Capsicum annuum* SAR8.2) genes were isolated by Lee and Hwang (2003) from a cDNA clone library prepared from HR lesions on pepper leaves infected with the Bv5-4a strain of *Xanthomonas campestris* pv. *vesicatoria (Xcv)*. These genes code for 86 amino acids and exhibit very high homology with each other (95–99%) at the amino acid level, and 43–50% homology with the corresponding proteins in tobacco.

All the special structural features of signal proteins are exhibited by the CASAR8.2A gene, which is not expressed in healthy plants, but accumulates rapidly and intensively in pepper leaves in response to the incompatible interaction. In pepper leaves infected with the virulent Ds1 strain of *X. campestris* pv. *vesicatoria* chlorotic and necrotic lesions are visible 6 days after infection. In contrast, very pronounced HR lesions are observed within 18 hours of infection on leaves infected with the avirulent Bv5-4a strain.

The expression of CASAR8.2A in response to *Colletotrichum coccodes* infection differs depending on the age of the plants, being greater in the 4-leaf stage than in the 8-leaf stage. The symptoms also differ between young and older plants; while the former are susceptible to *C. coccodes* infection, resistance is seen to be developing in the latter.

Phytophthora capsici induces CASAR8.2A accumulation in the stems of pepper plants in response to both the compatible and incompatible interaction. Infection of the lower leaves with *Xcv* also induces CASAR8.2A systemically in the upper leaves.

Abiotic elicitors and plant hormones such as salicylic acid, DL-β-amino-*n*-butyric acid, benzothiadiazol, ethylene, methyl jasmonate, indoleacetic acid and abscisic acid (ABA) also induce the CASAR8.2A gene.

Xcv infection results not only in CASAR8.2A transcripts, but also in PR-1 mRNAs and ethylene biosynthesis. The latter is directly proportional to the accumulation of PR protein. As both ethylene and methyl jasmonate lead to the rapid accumulation of *CASAR8.2A* transcripts in pepper leaves, they can be assumed to be signal molecules capable of activating the SAR8.2 gene. Irrespective of the concentration, treatment with methyl jasmonate causes the production of *CASAR8.2A* mRNA. The CASAR8.2A gene is also activated by a high salt concentration and by cold and drought stress, but not by injury.

CASAR8.2A transcripts, like those of thionin and the PR proteins chitinase and β-1,3-glucanase, are localised in the epidermal cells and in the phloem. The task of the SAR8.2 proteins is to act as a defence barrier for the easily damaged phloem. *CASAR8.2A* also generates signal molecules as a response to pathogen penetration, which activate further defence responses in neighbouring tissues.

As the CASAR8.2A gene is induced by all the biotic and abiotic stress factors tested so far, with the exception of injury, it is a useful marker of systemically induced biotic and abiotic resistance in pepper, making it suitable for the signalling of pathogen infection, abiotic elicitors and environmental stress (Lee and Hwang, 2003).

Thionins

Thionins are small, toxic proteins containing cysteines linked by internal disulphide bonds, which are involved in numerous biochemical reactions in infected tissues. Their primary role is in the compatible and incompatible interaction with pathogenic fungi (Bohlmann and Apel, 1991), but they are also effective against Gram-positive and Gram-negative bacteria and against yeasts (Florack and Stiekema, 1994). They are found in plants in the seed endosperm, the stems and roots and in etiolated or pathogen-stressed leaves (Bohlmann and Apel, 1991), while at cell level they can be detected in the vacuoles and cell-wall (Bohlmann et al., 1988). The constitutive expression of thionins in the roots could be related to the inhibition of pathogen penetration (Gu et al., 1992).

CATHION1 is a pepper gene activated in response to infection by various pathogens and is constitutively expressed in pepper roots and flowers. In the case of infection with *Colletotrichum coccodes*, the most dangerous fungal disease of pepper, causing spots on the fruit, there is a very high level of CATHION1 transcript accumulation in the flowers. Some species of the *Colletotrichum* genus are capable of responding to the physical and chemical signals given by the plant by producing appressoria, which mask these signals. The pathogenicity of these fungi, and probably their host-specificity too, is determined in an early stage of infection. The thionin genes appear to be involved in defence, but their role in the flowers and roots has not yet been clarified. In leaves infected with *C. coccodes* intense *CATHION1* activity can be observed, particularly in young plants; the mRNA accumulation is much more intense at the 4-leaf stage than at the 8-leaf stage. This probably indicates that the expression of the CATHION1 gene is linked with the development of the disease rather than with the resistance response. In the leaves transcripts can be detected in phloem cells and in the infection region, suggesting the specific activity of gene products in infected host cells. A high level of thionin in the infected region generates the formation of signal molecules in the pathogen, leading to further responses in neighbouring plant tissues (Lee et al., 2000).

The CATHION1 mRNA accumulation in green fruit is much more intense than in ripe red fruit in response to *Colletotrichum gloeosporioides* infection. Spots can be observed on the green fruits five days after infection, while no symptoms are visible on red fruits even after seven days. The fungus is able to penetrate the epidermis cells in green fruits, but not in red ones. The rapid activation of CATHION1 in infected green fruits is probably related with the development of the disease caused by *C. gloeosporioides* (Lee et al., 2000).

Proteinase inhibitors

In the digestive system of animals, proteinase inhibitors (PI) inhibit the hydrolysis of peptide bonds by chymotrypsin and other trypsins. Numerous plant species accumulate proteinase inhibitors in their seeds or vegetative organs, where they probably play a role in the defence against insect pests (Green and Ryan, 1972; Koiwa et al., 1997). PI expression may be constitutive, but may also be induced by various environmental effects such as injury, bacterial infection, plant hormones or fungal elicitors. These proteins are also capable of inhibiting a wide range of proteinases of microbial and animal origin, and a small number of plant proteinases (Ryan, 1990).

The proteinase inhibitor II gene (*CaPI-2*) isolated by Kim et al. (2001) from pepper exhibits 47% and 53% homology with the corresponding genes of tobacco and tomato, respectively, at the amino acid level. The primary structure, 304 amino acids in length, contains what is assumed to be a signal peptide and three highly conserved repeated proteinase inhibitor domains with one reactive trypsin-specific and two chymotrypsin-specific sites. The *CaPI-2* mRNA is uniformly expressed in the leaves, stem and roots of healthy pepper plants. In response to mechanical injury gene expression is intensified in the neighbourhood of the injury and even in more distant tissues. *CaPI-2* is expressed both locally and systemically in the leaves and stem as the result of injury, while it is only locally expressed in the roots. It is known that genes activated by ABA can also be induced by salt or polyethylene glycol (PEG) treatment. *CaPI-2* expression is intensified by treatment with exogenous ABA or salt or as the result of electroshock, but neither PEG nor cold stress caused any detectable mRNA accumulation.

Fibrillin

Fibrillin is a widely occurring protein, particularly frequent in chromoplasts containing fibril (fibrous structures that store carotenoids). In chromoplast fibril the fibrillin molecules are directly connected to the plastid stroma and interact internally with the polar head groups of phospholipids and galactolipids. The carotenoids are located in the core, surrounded by these lipids (Deruère et al., 1994). In the chloroplasts the carotenoids are components of photosynthetic complexes and play a role in light absorption and in preventing the formation of oxygen radicals (Frank and Cogdell, 1993). Carotenoid pigments are accumulated in the non-photosynthesising chromoplasts and cause the yellow, orange and red coloration of the fruit and flowers. These carotenoids are stored in fibrils consisting of polar lipids, carotenoids, tocopherol and fibrillin (Deruère et al., 1994).

Very active carotenoid synthesis occurs during the ripening of pepper, resulting in the characteristic red fruits. It is clear from the investigations of Chen et al. (1998), however, that the *fib* gene controlling fibrillin production, previously thought to be specific to the chromoplast, is induced not only in this cell organelle. The *fib* gene is activated by injury and drought stress and also accumulates in the leaves. In response to drought stress or injury the level of *fib* mRNA and fibrillin in pepper leaves increases within 24 hours and this enhanced expression is maintained for several days. The effect of stress on the expression of the *fib* gene is clearly illustrated by the fact that this expression is far more intense in severely dehydrated plants than in those with a milder level of dehydration. In non-stressed leaves the *fib* gene activity is extremely low and can only be detected at the protein level, not by RNA hybridisation. In response to stress, *fib* transcription is activated fairly slowly, as it can still not be detected six hours after exposure to stress, or in the affected regions after treatment leading to rapid cell death. The herbicide paraquat, which generates superoxide anions in Photosystem I, also induces *fib* activity in the leaves, but only in the light. It can be concluded from the above that, if *fib* is to be expressed, a complex signalling system must first develop, which then activates the gene. This signalling system probably works as follows: injury indirectly causes photosynthesis repression, leading in the presence of light and oxygen to the formation of various ROS, such as superoxide ions, which induce photooxidative stress. The signal transduction induced by this stress from the chloroplasts to the nucleus then activates the *fib* gene. The signal transduction mechanism must also include a regulatory link between the original external stress and the nucleus through the fibrillin-containing plastid.

Stellacyanin

Stellacyanin is a protein responsible for electron transfer in the photosynthetic system and was isolated from the latex of *Rhus vernicifera* (Reinhammar, 1970). Stellacyanin is induced in plants in response to biotic and abiotic stress factors such as drought, high salt content and abscisic acid treatment.

The *CASLP1* (*C. annuum* stellacyanin-like protein) gene was isolated by Kong et al. (2002) from a cDNA clone library originating from *Xcv*-infected pepper leaves. The *CASLP1* gene is expressed in pepper leaves in response to *Xcv* infection in the case of both the compatible and incompatible interaction, but is not extressed constitutively in healthy organs. In the incompatible interaction intense mRNA accumulation can be detected within 18 hours of infection, with the simultaneous appearance of the hypersensitive reaction in infected leaves. This is followed by a drastic rise in the accumulation of transcripts, with a maximum 30 hours after infection. In the case of the compatible interaction, however, the *CASLP1* mRNA accumulation gradually decreases between 18 and 30 hours after infection.

Twelve hours after *C. coccodes* infection weak *CASLP1* gene induction is observed in pepper leaves, reaching a maximum 48 hours after infection in 4-leaf plants, while in the 8-leaf stage there is a sudden increase in the transcript quantity after 72 hours. At the same time, the symptoms are much more severe in younger plants, while the resistance of the plants to *C. coccodes* increases as they develop.

In the case of *P. capsici* infection the *CASLP1* gene is induced in both the compatible and the incompatible interaction, though the typical symptoms of phytophthora infection are not observed in the latter case. *CASLP1* transcripts are primarily accumulated in the phloem cells and in the vascular bundles of the stem, as are thionin, the PR-1 protein and chitinase.

The *CASLP1* gene is induced earlier in ripe fruits infected with *C. gloeosporioides* than in green fruits, and the accumulation of transcripts is particularly high in green, immature fruits. Symptoms are observed on green fruits four days after infection, but not on ripe fruits. *CASLP1* transcripts can also be detected in the phloem cells of pepper plants infected with *C. gloeosporioides*.

Infection studies indicate that the activity of the *CASLP1* gene is not part of the systemically induced defence, but is limited to the site of infection.

The only abiotic elicitor found to induce intense *CASLP1* gene expression is methyl jasmonate, while the gene is not activated by treatment with salicylic acid, ethylene, DL-β-amino-*n*-butyric acid (BABA) or benzothiodiazol (BTH).

In response to injury the activity of the *CASLP1* gene differs from that described above. In 2-leaf plants transcripts can be detected in the injured lower leaf 3 hours after injury, while in the uninjured upper leaf intense accumulation can also be observed 4, 8 and 12 hours after the lower leaf was injured. This same type of gene expression can be induced in pepper by MeJA treatment, which is known to have a strong inducing effect on systemic injury response genes.

The expression of the *CASLP1* gene is gradually induced in the leaves after treatment with exogenous ABA, while expression is strong in the stem an hour after treatment and then remains at this level, suggesting that a stress signal is transmitted from the lower to the upper leaves in response to ABA treatment.

In contrast to ABA treatment, the *CASLP1* gene activity in response to NaCl treatment is much more intense in the leaves than in the stem, which can probably be attributed to organ-specific gene expression induced by various control mechanisms.

The *CASLP1* gene is also strongly induced by drought in the stem and leaves of young pepper plants.

Pathogen-induced transcription factor

Transcription factors are bound to specific transcription-regulating sites in the promoter or enhancer region of the DNA. The transcription factors can be selectively activated or deactivated by other proteins, often as the last step in signal transduction. Although the transcription activation of genes involved in the development of resistance is fundamental for the response to pathogen infection, very few transcription factors that react directly and specifically to pathogens have yet been identified (Rushton and Somssich, 1998).

The *PPI1* gene isolated from pepper by Lee et al. (2002) is expressed in the incompatible interaction with strain 61 of the *P. syringae* pv. *syringae* virus, causing mild spottedness in pepper, and with the *Xcv 3* strain. In yeast PPI1 is a nuclear protein that activates transcription. In plants the PPI1 protein is a transcription factor involved in the defence response to pathogen infection.

Interestingly enough, the *PPI1* gene is only expressed in response to biotic stress, while it is not induced by abiotic stress factors, such as treatment with salicylic acid, methyl jasmonate, ethylene, H_2O_2 or ABA. This is surprising because basic leucine zipper (bSIP) DNA-binding proteins can be induced by plant hormones such as ABA, and by other environmental factors such as low temperature, light or injury. It follows from this that the *PPI1* gene is not required for the induction of *PR* genes and is probably part of a defence signalling system independent of the better-known salicylic acid, ethylene and methyl jasmonate pathways.

TMV resistance

In pepper the L^1, L^2, L^3 and L^4 genes determine resistance to tobacco mosaic virus (TMV). Based on their interaction with the resistance genes, the TMV viruses are classified into the P_0, P_1, $P_{1.2}$ and $P_{1.2.3}$ pathotypes, which are capable of infecting plants containing the following L genes:

P_0 – HR: L^1, L^2, L^3, L^4;
P_1 – systemic: L^1; HR: L^2, L^3, L^4;
$P_{1.2}$ – systemic: L^1, L^2; HR: L^3, L^4;
$P_{1.2.3}$ – systemic: L^1, L^2, L^3; HR: L^4 (Boukema, 1982).

In experiments carried out by Shin et al. (2001) infection with the P_0 pathotype of TMV induced HR in the pepper variety Bukang. Infection with the avirulent P_0 pathotype caused a significant increase in the expression level of at least 16 genes, while after infection with the virulent $P_{1.2}$ type the expression of these genes remained unchanged. The sequences of these genes or their derived amino acid sequences exhibited homology with known proteins exhibiting enhanced activity in response to virus infection in higher plants. Six of the 16 derived proteins, an alanine aminotransferase, an enolase, an NADP-malate enzyme, a pyruvate decarboxylase, a β-ketoacyl-ACP synthase and an ornithine decarboxylase enzyme, are involved in metabolic processes. Alanine aminotransferase, enolase and the NADP-malate enzyme have a role in pyruvate production, which then forms part of the ethanol biosynthetic pathway. The activity of these genes is substantially greater in leaves exhibiting HR compared with that in leaves not infected with TMV. These results confirm observations in other plant/virus systems, suggesting that sudden changes occur in the metabolism in the altered environment resulting from HR in response to pathogen attack.

In addition to the above, the activity of the gene coding for ornithine decarboxylase, which is involved in endogenous polyamine production, also increases considerably in leaves infected with TMV and exhibiting necrotic lesions. Polyamines play a role in stimulating the expression of PR genes and thus in the development of TMV resistance. One characteristic feature of the plant–pathogen interaction is the involvement of PR proteins, of which at least 14 families exist. Genes coding for PR proteins are expressed in the course of pathological events such as the resistant, hypersensitive or susceptible responses. Among the pepper PR genes, intense activity is exhibited by PR1, PR4, PR10 and those coding for SAR8.2 protein precursors in the course of HR in response to infection with the TMV-P_0 pathotype, while their activity is extremely weak after infection with the TMV-$P_{1.2}$ pathotype. This suggests that resistant plants recognise the virus promptly and are able to activate defence responses by stimulating the transcription activity of PR protein genes.

In response to pathogenic attack a complex defence system is activated in plants. This involves not only the activation of PR and antimicrobial genes, but also the strengthening of the cell-wall through lignin deposits (Dean and Kúc, 1987) and oxidative cross-bonding (Brisson et al., 1994). Unlike bacterial and animal viruses, plant viruses are faced with the task of penetrating the physical barrier represented by the cell-wall. They solve this problem using movement proteins (MP) which allow them to migrate from cell to cell through the cell-wall in an infected leaf until they reach the phloem. From there they are capable of travelling great distances through the vascular bundles, thus infecting the whole plant systemically (Hull, 1991).

Gene *CaTin2*, induced in pungent pepper in response to TMV-P_0 infection and coding for a 23 kDa cell-wall protein, was isolated by Shin et al. (2001) and functionally characterised by Shin et al. (2003b). *CaTin2* accumulates mainly in the roots and leaves, with small quantities in the flowers, while none could be detected in the fruit or stem. The accumulation of *CaTin2* transcripts begins 6 hours after infection and increases continually for 48 hours if the L^2 resistance gene is present. In response to TMV-$P_{1.2}$ infection *CaTin2* is not expressed until 48 hours after infection in the same pepper line, indicating that *CaTin2* is expressed in plants exhibiting the HR response to TMV infection.

Three days after infecton *CaTin2* is also accumulated in leaves far from the site of infection.

CaTin2 is not induced by infection with the bacterium *Xanthomonas campestris* pv. *vesicatoria*, suggesting that it is only activated in the virus–plant interaction.

The gene can, however, be induced by various elicitors, such as salicylic acid, ethylene, methyl jasmonate, abscisic acid or high salt content, though after injury the transcription level is extremely low and the gene is expressed mainly in the immediate neighbourhood of the injury.

The role of the *CaTin2* gene in virus resistance is demonstrated by the fact that transgenic tobacco plants containing the gene are resistant to TMV and CMV infection, while the resistance level of antisense plants does not change.

With regard to its structure, *CaTin2* does not exhibit homology with any known genes except at the 5' end, which contains a cell-wall signal peptide and an incomplete glycine-rich motif.

Promoters are structural components that regulate gene transcription. In most cases they are one-way, though exceptions, two-way promoters, are also known. In plants the latter are primarily chloroplast genes (Schwarz et al., 1981; Berends et al., 1987; Koichi et al., 1988; Meng et al., 1991), though on occasion they may be nuclear genes (Keddie et al., 1994; Sadanandom et al., 1996). One example is the *CaTin1* and *CaTin1-2* genes induced by TMV infection in pepper (Shin et al., 2003a), which are connected by a two-way promoter. The nucleotide sequences of the two genes exhibit 79% homology, but are not homologous to any other gene in the pepper genome. Transcripts of these genes are only found in abundance in the roots. *CaTin1* is induced somewhat earlier than *CaTin1-2*.

In response to *Xanthomonas campestris* pv. *vesicatoria* infection the *CaTin1-2* gene is accumulated in pepper plants carrying the Bs2 resistance gene, indicating that this gene can be induced not only by viruses, but also by bacterial infection. However, the *CaTin1-2* gene is not induced by either salicylic acid or methyl jasmonate treatment. Gene expression can be observed for around 8 hours in response to ethylene, and salt stress also induces only moderate gene activity, but methyl viologen treatment results in substantial gene induction. Methyl viologen induces hydrogen peroxide, which may be decisive for the expression of *CaTin1-2*, as in the case of other genes. The expression of *CaTin1* and *CaTin1-2* is identical in the above cases, except that in the case of TMV-P_0 infection *CaTin1* is expressed more rapidly. The explanation for this almost identical gene expression is probably the common promoter of the two genes, while the more rapid expression of *CaTin1* could be due to the fact that the elicitor sensor is closer to this gene.

The promoter is probably responsible for the fact that, under normal circumstances, *CaTin1-2* is only expressed in the roots. Few data are as yet available on the regulation of root-specific gene expression. Only a small number of root-specific promoters are known, and in most cases the activity of these is restricted to a certain part of the root. The only *cis*-functioning root-specific sequences are the *ocs* elements, the *as-1* elements and the AT-rich regions. Two such AT-rich regions are present in *CaTin1-2*.

Virus resistance by post-transcriptional gene silencing

Like other plants, pepper also has a defence mechanism against virus infection that is capable of recognising and degrading "alien" RNA if it accumulates beyond a certain level, a process referred to as post-transcriptional gene silencing (PTGS). In the course of this process the endogenous RNA-dependent RNA polymerase produces short complementary RNA (cRNA) molecules from specific RNAs serving as templates. These cRNAs then spread through the cytoplasm and bind to complementary sequences. These short dsRNA (double-stranded RNA) segments act as alignment points for RNA-degrading enzyme activity. The RNAs that initiate PTGS have some sort of aberration, for example their transcription finishes earlier than normal, making them shorter and deficient in information (Lindbo et al., 2001).

In addition to its other biological roles, PTGS also represents a defence strategy against viruses for the plants (Waterhouse et al., 1999). In the course of the defence mechanism the plant recognises the alien virus RNA and stimulates a selectively degrading PTGS response. Nevertheless, many viruses are capable of repressing PTGS and in some cases compatible viruses develop some form of cross-protection if they have common nucleotide sequences. The importance of PTGS in virus resistance is demonstrated by the fact that *Arabidopsis* mutants in which PTGS is non-functional become hypersensitive to certain viruses. Most plant viruses possessing an RNA genome are replicated via an intermediate dsRNA phase which, however, induces a PTGS response. Like the plant viruses, the PTGS activated in a few cells is capable of migrating over short or long distances. In order to reach a large number of cells, the hypothetical PTGS nucleic acid signal probably goes through intensifying and translocational phases. This ability of the PTGS signal to migrate over short or long distances allows plants to protect non-infected cells against viral attack.

Xanthomonas *resistance*

Bacterial spots are caused in pepper by *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*). At locations with high humidity and abundant rainfall *Xcv* may completely destroy the crop. Sources resistant to the bacterium are known in pepper, but chemicals are essential to protect susceptible varieties. Genes *Bs1, Bs2* and *Bs3* have been identified in pepper, and the corresponding avirulence genes, *avrBs1, avrBs2* and *avrBs3* have been cloned from *Xcv*. The significance of these genes in the development of resistance has also been demonstrated. The genetic interaction between the avirulence gene *avrBs2* and the resistance gene *Bs2*, derived from the wild species *Capsicum chacoense*, is of especial interest. The avrBs2 protein probably possesses phosphodiesterase properties. *Xcv* strains carrying a mutation that inactivates the *avrBs2* gene are less virulent even to susceptible pepper lines, growing 10–100 times more slowly than the wild type. Pepper lines carrying the *Bs2* resistance gene are resistant to both the virulent and avirulent strains of *Xcv*. In some hybrid lines, which are heterozygous for the *Bs2* locus, resistance may be overcome under field conditions (Mudgett et al., 2000; Tai et al., 1999).

Tai et al. (1999) isolated the *Bs2* gene using a positional cloning strategy and the open reading frame (ORF) was identified by sequencing. The protein probably coded by the ORF exhibits great homology with the NBS-LRR (nucleotide binding site – leucine-rich repeat) class of R genes. A short intron and an extremely long, 27 kb intron were found in the 5'-end untranslated region, located close to the end of the coding region. The protein probably coded by *Bs2* exhibited the greatest homology with the PVX resistance gene of potato (38%).

The resistance of the *Bs2* gene was tested by transforming it into tomato plants by means of *Agrobacterium tumefaciens* mediation. The growth of the bacterium was then examined in the transgenic plants, where the *Bs2* gene was found to inhibit both the growth of *Xcv* strains expressing the *avrBs2* gene and the appearance of symptoms on the leaves. These results confirm the usefulness of the *Bs2* gene in developing pepper plants with resistance to bacterial disease.

In the course of an *Agrobacterium*-mediated, transient coexpression analysis, an *A. tumefaciens* strain containing a CaMV 35S-*avrBs2* construct and a strain containing a 35S-*Bs2* candidate gene were injected into various plants. When only one of the constructs was injected into susceptible pepper plants, no resistance response was observed. However, when they were applied together, hypersensitive symptoms were visible, similar to those given by pepper plants carrying the *Bs2* gene. The same could be observed on other *Solanaceae*, such as potato and aubergine, while no HR symptoms were exhibited by species from other families, such as *Arabidopsis*, *Petroselium*, cucumber and broccoli. These results suggest that the *Bs2* gene only functions in *Solanaceae*.

Resistance gene homology between pepper and other members of the Solanaceae

It is known from the work of Livingstone et al. (1999) and Grube et al. (2000) that there is a high degree of similarity between various genera of the *Solanaceae* family at the DNA level, indicating that no significant genome loss has occurred since the genera became distinct. As a consequence of translocations, para- and pericentric inversions, and the combination/separation of genomic regions, around thirty rearrangements caused by chromosome breakage distinguish the genomes of tomato, potato and pepper. The pepper homologues of cloned resistance genes with known functions are found in positions syntenic to those in other *Solanaceae* genomes. In a few cases the genes are mapped in phenotypically determined positions. The pepper homologues of the N gene providing TMV resistance in tobacco are in positions equivalent to the Ry_{sto}/Ry_{adg}, *Rmci* and *Sen1* R genes in potato. The position of the *Sw-5* pepper homologues is equivalent to that of *Sw-5* in tomato, Nx_{phu} in potato and the *cmv3.1* QRL (quantitative resistance locus) in pepper. A further *Sw-5* homologue can be found near the *Cm7.1* QRL in tomato. Due to the great sequence homology between the *Pto* and *Fen* genes it is difficult to distinguish between these genes on the map. *Pto/Fen* homologues can be found in at least five genomic regions in pepper. One of these homologues is found in the position of the *Prf*, *Pto* and *Fen* genes in tomato and of a *Pto* gene in potato. The other pepper *Pto/Fen* homologues were mapped to an extended group of R genes, including *Grp1, phyt3, Gpa, Pi01, R1, Rx-2* and *Nb*, in potato, and to the R gene group *Cm6.1, Mi, Ty-1, Ol-1, Cf-2* and *Cf-5* in tomato.

All the pepper R gene homologues investigated up till now are found in syntenic regions in the original genus. In some cases these genes also occupy unique positions either in the same genus or in other genera. The comparative mapping data obtained so far indicate that the same pathogen resistance is controlled in different genera by non-homologous genes. Non-homologous R genes are probably capable of starting the same conserved response avalanche, thus explaining why the resistance response may be phenotypically similar in various genera even though there is no genetic relationship between the loci.

When the species became differentiated, the R genes largely retained their chromosomal positions.

While the taxonomic specificity of the R genes may develop rapidly, the general functions of the R alleles, such as the induction of the resistance response, were probably conserved in homologous loci in related plant genera. While one homologue of a single gene may correspond exactly to the phenotypic position of a different pathogen resistance in another host plant, no R genes have yet been identified where the homologues co-segregate with similar pathogen resistance traits in another genus.

Based on the rapid increase in sequence information, knowledge on R genes is expected to expand greatly in the near future. Comparative analyses within plant families should make it possible to reveal processes regulating the functioning and development of this important gene family.

Pepper genes causing allergy

Some 30–50% of people today are allergic to natural latex, an allergy that is sparked by certain foods of plant origin, particularly the consumption of fresh fruit (Wagner and Breiteneder, 2002). Among the commonest fruit and vegetables these include potato, tomato, pepper, banana and peach. The causes of the allergic reaction are human antibodies that recognise structures of various plant proteins that are phylogenetically closely related or conserved in the evolutional sense. A number of plant proteins causing latex allergy have already been identified. Two of these are defence proteins also found in pepper, one a class I chitinase, the other a β-1,3-glucanase.

Willerroider et al. (2003) isolated the Capa2 gene, a cDNA coding for profilin, from pepper. Profilins are intracellular plant proteins that cause allergic symptoms in 20% of people allergic to plant foodstuffs. Profilins may be found not only in the fruit, but also in the pollen, thus playing a role in the development of pollen sensitivity as well as in food allergies.

Mobile genetic elements in pepper

Mobile genetic elements, or transposons, are small mobile sequences in the genome, which can be divided into two types depending on the mode of transposition:

– Retrotransposons are first transcribed into RNA, from which DNA is formed by reverse transcription. This DNA molecule exists as an independent molecule outside the genome, and may be incorporated either into the original or into another chromosome when it re-enters the genome. In either case, the result will be two copies of the same element at different sites in the genome.

– DNA transposons do not require an intermediate RNA transcription/reverse transcription step, but are either directly copied into the target sequence or leave their original place in the genome and are incorporated elsewhere.

Retrotransposons are extremely frequent in eukaryotes, while DNA transposons are substantially rarer. Transposition is catalysed by enzymes known as transposases, which are usually coded by the transposons themselves.

There is an extremely large proportion of mobile genetic elements in pepper. Some scientists consider that this vast number of retrotransposons can be explained by the fact that the pepper genome is 3–4 times larger than that of tomato (Livingstone et al., 1999).

Transposons

A small, unique family of transposons known as *Alien*, consisting of inverted repeats, are frequently found in the pepper genome, where the *Alien* elements exhibit great homology. They are characterised by a 28 bp terminal inverted repeat (TIR) that, due to its limited coding capacity, does not allow specifically recognisable transposase to be coded. It is thus probable that their mobility is ensured by a transposase found at another chromosomal locus. *Alien* elements can also be found in the 5'-end region of the phosphorylase and patatin genes in potato starch. It is interesting to note the great homology between the TIR sequences of the extrachromosomal linear pSKL DNA plasmid of *Saccharomyces kluyveri* and the TIR of *Alien* elements. There is known to be a protein bound to the TIRs of the pSKL plasmid that is required for the autoreplication of the linear DNA molecule, which may take place through a mechanism similar to the replication of the DNA molecules of the *Bacillus subtilis* bacteriophage Ø29. In plants the functional and evolutional role of these sequences has not yet been clarified (Pozueta-Romero et al., 1995).

Ts1 elements

Mobile genetic elements are thought to play an important role in the diversification and evolution of the nuclear genome and in the endosymbiosis of the organelles (Nugent and Palmer, 1991). (According to the endosymbiosis theory, the mitochondria and chloroplasts of eukaryotic cells originated from symbiotic prokaryotes.)

Retrotransposons consist of virus-like and non-virus-like types. The latter include the retropseudogenes, LINEs (long interspersed repetitive elements) and SINEs (short interspersed repetitive elements). LINEs contain a reverse transcriptase-like gene, but SINEs do not. Nevertheless, SINEs are also capable of transposition with the help of reverse transcriptases from other retroelements. Three SINE families are known in plants: *Ts*, *p-SINE1* and $S1_{Bn}$.

As pepper fruits ripen, the expression of the *PAP* gene, that codes for a plastid-lipid associated protein, intensifies greatly. The coding sequence is separated by one 805 bp and one 95 bp intron in the genomic DNA. In pepper the analysis of the *PAP* mRNAs led to the isolation of a mobile genetic element belonging to the *Ts* family of SINEs, that causes transcription errors in the *PAP* gene as the result of cis-functioning sequences. These are probably the first tRNA SINEs to be isolated from plants.

In contrast to plants, SINEs are extremely frequent in the animal kingdom. In relation to the SINEs mentioned above, some authors suggest that the *Ts* elements may have been transferred from animals to plants by horizontal transmission (Pozueta-Romero et al., 1998).

Extrachromosomal genes

In addition to nuclear genes, plastids and mitochondria also possess genetic material in plants. These organelle genes are generally inherited maternally through the egg-cell. A knowledge of the photosynthesis genes found in the chloroplasts is extremely important in herbicide research. Information on mitochondrial genes is of greatest significance in the production of hybrids.

Cytoplasmic male sterility (CMS) is the result of either mutations in the mitochondrial genome or incompatibility between the nucleus and the mitochondria. Structural differences between fertile and sterile mitochondrial DNA (mtDNA) are the result of rearrangements due to the recombination of homologous sequences. In the course of this process various parts of the mtDNA may be restructured, generating new, chimeric genes related to CMS. The sterile phenotype is usually associated with the production of proteins from chimeric genes. The incorporation of these proteins into the mitochondrial membrane or multiprotein enzyme complexes results in damage to mitochondrial functions.

Cytoplasmic male sterility in pepper was first described by Peterson (1958). Very little information on pepper CMS is yet available at the molecular level. One 20-kDa protein is known to be involved in the expression of CMS, and in one type of pepper CMS the mitochondrial genes *coxII* and *atp6* carry a mutation compared with the fertile type. These mutations are found downstream of the 3' end in *coxII* and upstream of the 5' end in *atp6*. In the fertile type the region between coxII and atp6 is about 30 kb in length, while in the CMS type it is only about 16 kb. This sequence shortening is fairly unusual in CMS plants; in other species the opposite is generally found (Kim et al., 2001).

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References

Albrecht, M., Klein, A., Hugueney, P., Sandmann, G., Kuntz, M. (1995): Molecular cloning and functional expression in *E. coli* of a novel plant enzyme mediating ξ-carotene desaturation. *FEBS Letters*, **372**, 199–202.

Bendahmane, A., Querci, M., Kanyuka, K., Baulcombe, D. C. (1999): Agrobacterium transient expression system as a tool for the isolation of disease resistance genes: application to the Rx2 locus in potato. *Plant J.*, **21**, 73–81.

Berends, T., Gamble, P. E., Mullet, J. E. (1987): Characterization of the barley chloroplast transcription units containing psaA-psbB psbD-psbC. *Nucl. Acids Res.*, **15**, 5217–5240.

Bohlmann, H., Apel, K. (1991): Thionins *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **42,** 227–240.

Bohlmann, H., Clausen, S., Behnke, H., Giese, H., Hiller, C., Reimann-Philipp, U., Schrader, G., Barkholt, V., Apel, K. (1988) Leaf-specific thionins of barley – a novel class of cell wall proteins toxic to plant-pathogenic fungi and possibly involved in the defence mechanism of plants. *EMBO,* **7,** 1559–1565.

Bohlmann, J., Stauber, E. J., Krock, B., Oldham, N. J., Gershenzon, J., Baldwin, I. T. (2002): Gene expression of 5-*epi*-aristolochene synthase and formation of capsidiol in roots of *Nicotiana attenuata* and *N. sylvestris*. *Phytochemistry*, **60**, 109–116.

Boukema, I. W. (1982): Resistance to TMV in *Capsicum chacoense* in *Capsicum* L. *Euphytica,* **29,** 433–439.

Bouvier, F., Backhaus, R. A., Camara, B. (1998a): Induction and control of chromoplast-specific carotenoid genes by oxidative stress. *J. Biol. Chem.*, **273**, 30651–30659.

Bouvier, F., d'Harlingue, A., Hugueney, P., Marin, E., Marion-Poll, A., Camara, B. (1996): Xanthophyll biosynthesis. Cloning, expression, functional reconstitution, and regulation of beta-cyclohexenyl carotenoid epoxidase from pepper (*Capsicum annuum*). *J. Biol. Chem.,* **271**, 28861–28867.

Bouvier, F., d'Harlingue, A., Suire, C., Backhaus, R. A., Camara, B. (1998b): Dedicated roles of plastid transketolases during the early onset of isoprenoid biogenesis in pepper fruits. *Plant Physiol.*, **117**, 1423–1431.

Brisson, L. F., Tenhaken, R., Lamb, C. (1994): Functions of oxidative cross-linking of cell wall structural proteins in plant disease resistances. *Plant Cell,* **6,** 1703–1712.

Chandler, P. M., Robertson, M. (1994): Gene expression regulated by abscisic acid and its relation to stress tolerance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.,* **45**, 113–141.

Chapple, C. (1998): Molecular-genetic analysis of plant cytochrome P450-dependent monooxygenases. *Annu. Rev. Plant Physiol. Plant Mol. Biol,.* **49**, 311–343.

Chen, H. C., Klein, A., Xiang, M., Backhaus, R. A., Kuntz, M. (1998): Drought- and wound-induced expression in leaves of a gene encoding a chromoplast carotenoid-associated protein. *Plant J.,* **14,** 317.

Cheng, C. M., Palloix, A., Lefebvre, V. (2002): Isolation, mapping and characterization of allelic polymorphism of *Chi3-P1*, a class III chitinase of *Capsicum annuum* L. *Plant Sci.*, **163**, 481–489.

Collinge, D. B., Slusarenko, A. J. (1987): Plant gene expression in response to pathogens. *Plant Mol. Biol.,* **9**, 389–410.

Curry, J., Aluru, M., Mendoza, M., Nevarez, J., Melendrez, M., O'Connell, M. A. (1999): Transcripts for possible capsaicinoid biosynthetic genes are differentially accumulated in pungent and non-pungent *Capsicum* spp. *Plant Sci.*, **148**, 47–57.

Dean, R. A., Kúc, J. (1987): Rapid lignification in response to wounding and infection as a mechanism for induced systemic protection in cucumber. *Physiol. Plant Pathol.*, **31**, 69–81.

Deruère, J., Romer, S., d'Harlingue, A., Backhaus, R. A., Kuntz, M., Camara, B. (1994) Fibril assembly and carotenoid overaccumulation in chromoplasts: a model for supramolecular lipoprotein structures. *Plant Cell.*, **6**, 119–133.

Deshpande, R. B. (1935): Studies in Indian chillies. 4. Inheritance of pungency in *Capsicum annuum* L. *Indian Jour. Agr. Sci.*, **5,** 513–516.

Do, H. M., Hong, J. K., Jung, H. W., Kim, S. H., Ham, J. H., Hwang, B. K. (2003): Expression of peroxidase-like genes, H_2O_2 production, and peroxidase activity during the hypersensitive response to *Xanthomonas campestris* pv. *vesicatoria* in *Capsicum annuum*. *Mol. Plant Microbe Interact.*, **16**, 196–205.

Dorey, S., Baillieul, F., Saindrenan, P., Fritig, B., Kauffmann, S. (1998): Tobacco class I and II catalases are differentially expressed during elicitor-induced hypersensitive cell death and localized acquired resistance. *Mol. Plant Microbe Interact.*, **11**, 1102–1109.

Egea, C., Alcázar, M. D., Candela, E. M. (1996): Capsidiol: Its role in the resistance of *Capsicum annuum* to *Phytophthora capsici*. *Physiol. Plant.*, **98**, 736–742.

Egea, C., Dickinson, M. J., Candela, M., Candela, E. M. (1999): β-1,3-Glucanase isoenzymes and genes in resistant and susceptible pepper (*Capsicum annuum*) cultivars infected with *Phytophthora capsici*. *Physiol. Plant.*, **107**, 312–318.

Ellis, J., Dodds, P., Pryor, T. (2000): Structure, function and evolution of plant disease resistance genes. *Curr. Opin. Plant Biol.*, **3**, 278–284.

Fahrendorf, T., Dixon, R. A. (1993): Stress responses in alfalfa (*Medicago sativa* L.) XVIII: Molecular cloning and expression of the elicitor inducible cinnamic acid 4-hydroxylase cytochrome P450. *Arch. Biochem. Biophys.*, **305**, 509–515.

Florack, D. E., Stiekema, W. J. (1994): Thionins: properties, possible biological roles and mechanisms of action. *Plant Mol. Biol.*, **26,** 25–37.

Frank, H. A., Cogdell, R. J. (1993): The photochemistry and function of carotenoids in photosynthesis. pp. 252–326. In: Young, A., Britton, G. (eds.), *Carotenoids in Photosynthesis.* Chapman and Hall, London.

Fujiwake, H., Suzuki, T., Iwai, K. (1982a): Capsaicinoid formation in the protoplast from the placenta of *Capsicum* fruits. *Agric. Biol. Chem.*, **46**, 2591–2592.

Fujiwake, H., Suzuki, T., Iwai, K. (1982b): Intracellular distributions of enzymes and intermediates involved in biosynthesis of capsaicin and its analogues in *Capsicum* fruits. *Agric. Biol. Chem.*, **46,** 2685–2689.

García-Pérez, M. D., Egea, C., Candela, M. E. (1998): Defence response of pepper (*Capsicum annuum*) suspension cells to *Phytophthora capsici*. *Physiol. Plant.*, **103,** 527–533.

Garcia-Pineda, E., Lozoya-Gloria, E. (1999): Induced gene expression of 1-aminocyclopropane-1-carboxylic acid (ACC oxidase) in pepper (*Capsicum annuum* L.) by arachidonic acid. *Plant Sci.*, **145,** 11–21.

Green, T. R., Ryan, C. A. (1972): Wound inducible proteinase inhibitor in plant leaves: a possible defense mechanism against insects. *Science*, **175**, 776–777.

Grube, R. C., Radwanski, E. R., Jahn, M. (2000): Comparative genetics of disease resistance within the *Solanceae*. *Genetics*, **155**, 873–887.

Gu, Q., Kawata, E. E., Morse, M. J., Wu, H. M., Cheung, A. Y. (1992): A flower-specific cDNA encoding a novel thionin in tobacco. *Mol. Gen. Genet.*, **234,** 89–96.

Ha, S. H., Kim, J. B., Hwang, Y. S., Lee, S. W. (2002): Molecular characterization of three 3-hydroxy-3-methylglutaryl-CoA reductase genes including pathogen-induced *Hmg2* from pepper (*Capsicum annuum*). *BBA – Gene Structure and Expression*, **1625**, 253–260.

Hirschberg, J. (2001): Carotenoid biosynthesis in flowering plants. *Curr. Opin. Plant Biol.*, **4**, 210–218.

Hong, J. K., Hwang, B. K. (2002): Induction by pathogen, salt and drought of a basic class II chitinase mRNA and its in situ localization in pepper (*Capsicum annuum*). *Physiol Plant.*, **114**, 549–558.

Hong, J. K., Jung, H. W., Kim, Y. J., Hwang, B. K. (2000): Pepper gene encoding a basic class II chitinase is inducible by pathogen and ethephon. *Plant Sci.*, **159**, 39–49.

Hugueney, P., Bouvier, F., Badillo, A., Quennemet, J., d'Harlingue, A., Camara, B. (1996): Developmental and stress regulation of gene expression for plastid and cytosolic isoprenoid pathways in pepper fruits. *Plant Phys.*, **111**, 619–626.

Hull, R. (1991): The movement of viruses within plants. *Semin. Virol.*, **2**, 89–95.

Hurtado-Hernandez, H., Smith, P. G., (1985): Inheritance of mature fruit color in *Capsicum annuum* L. *J. Hered.*, **76**, 211–213.

Jones, J. (1996): A kinase with keen eyes. *Nature*, **385**, 397–398.

Joos, H-J., Hahlbrock, K. (1992): Phenylalanine ammonia-lyase in potato (*Solanum tuberosum* L.) – genomic complexity, structural comparison of two selected genes and modes of expression. *Eur. J. Biochem.*, **204**, 621–629.

Jung, W. H., Hwang, B. K. (2000): Pepper gene encoding a basic β-1,3-glucanase is differentially expressed in pepper tissues upon pathogen infection and ethephon or methyl jasmonate treatment. *Plant Sci.*, **156**, 23–34.

Keddie, J. S., Tsiantis, M., Piffanelli, P., Hatzopoulos, P., Murphy, D. J. (1994): A seed-specific *Brassica napus* oleosin promoter interacts with a G-box-specific protein and may be bi-directional. *Plant Mol. Biol.*, **24,** 327–340.

Kellogg, B. A., Poulter, D. C. (2002): Chain elongation in the isoprenoid biosynthetic pathway. *Curr. Opin. Chem. Biol.*, **1,** 570–578.

Kim, S., Hong, Y. N., An, C. S., Lee, K. W. (2001): Expression characteristics of serine proteinase inhibitor II under variable environmental stresses in hot pepper (*Capsicum annuum* L.). *Plant Sci.*, **161**, 27–33.

Kim, Y. J., Hwang, B. K. (1994): Differential accumulation of β-1,3-glucanase and chitinase isoforms in pepper stems infected by compatible and incompatible isolates of *Phytophthora capsici*. *Physiol. Mol. Plant Pathol.*, **45**, 195–209.

Kim, D. H., Kang, J. G., Kim, S., Kim, B. D. (2001): Identification of *cox*II and *atp*6 regions as associated to CMS in *Capsicum annuum* by using RFLP and long accurate PCR. *J. Kor. Soc. Hort. Sci.*, **42**, 121–127.

Koichi, T., Yoshida, T., Komano, T. (1988): Divergent mRNA transcription in the psbB operon. *EMBO*, **7**, 885–891.

Koiwa, H., Bressan, R. A., Hasegawa, P. M. (1997): Regulation of protease inhibitors and plant defense. *Trends Plant Sci.*, **2,** 379–384.

Kombrink, E., Somssich, I. E. (1995): Defense responses of plants to pathogens. *Adv. Bot. Res.*, **21**, 1–34.

Kong, H. Y., Jung, H. W., Lee, S. C., Choi, D., Hwang, B. K. (2002): A gene encoding stellacyanin is induced in *Capsicum annuum* by pathogens, methyl jasmonate, abscisic acid, wounding, drought and salt stress. *Physiol. Plant.*, **115**, 550–562.

Kwon, S. I., An, C. S. (2001): Molecular cloning, characterization and expression analysis of a catalase cDNA from hot pepper (*Capsicum annuum* L.). *Plant Sci.*, **160**, 961–969.

Lee, B., Choi, D., Lee, K-W. (1998): Isolation and characterization of *o*-diphenol-*O*-methyltransferase cDNA clone in hot pepper (*Capsicum annuum* L.). *J. Plant Biol.*, **41**, 9–15.

Lee, S. C., Hwang, B. K. (2003): Identification of the pepper SAR8.2 gene as a molecular marker for pathogen infection, abiotic elicitors and environmental stresses in *Capsicum annuum*. *Planta*, **216**, 387–396.

Lee, S. C., Lee, Y. K., Kim, K. D., Hwang, B. K. (2000): *In situ* hybridization study of organ- and pathogen-dependent expression of a novel thionin gene in pepper (*Capsicum annuum*). *Physiol. Plant.*, **110**, 384.

Lee, S. J., Lee, M. Y., Yi, S. Y., Oh, S. K., Choi, S. H., Her, N. H., Choi, D., Min, B. W., Yang, S. G., Harn, C. H. (2002): PPI1: a novel pathogen-induced basic region-leucine zipper (bZIP) transcription factor from pepper. *Mol. Plant Microbe Interact.*, **15**, 540–548.

Lefebvre, V., Kuntz, M., Camara, B., Palloix, A. (1998): The capsanthin-capsorubin synthase gene: a candidate gene for the y locus controlling the red fruit colour in pepper. *Plant Mol. Biol.*, **36**, 785–789.

Lindbo, J. A., Fitzmaurice, W. P., della Cioppa, G. (2001): Virus-mediated reprogramming of gene expression in plants. *Curr. Opin. Plant Biol.*, **4**, 181–185.

Livingstone, K. D., Lackney, V. K., Blauth, J. R., van Wijk, R., Jahn, M. K. (1999): Genome mapping in *Capsicum* and the evolution of genome structure in the *Solanaceae*. *Genetics*, **152,** 1183–1202.

Macho, A., Lucena, C., Sancho, R., Daddario, N., Minassi, A., Muñoz, E., Appendino, G. (2003): Non-pungent capsaicinoids from sweet pepper. Synthesis and evaluation of the chemopreventive and anticancer potential. *Eur. J. Nutr.*, **42,** 2–9.

Malamy, J., Klessig, D. F. (1992): Salicylic acid and plant disease resistance. *Plant J.*, **2**, 643–654.

Meng, B. Y., Wakasugi, T., Sugiura, M. (1991): Two promoters within the psbK-psbI-trnG gene cluster in tobacco chloroplast DNA. *Curr. Genet.*, **20,** 259–264.

Mudgett, M. B., Chesnokova, O., Dahlbeck, D., Clark, E. T., Rossier, O., Bonas, U., Staskawicz, B. J. (2000): Molecular signals required for type III secretion and translocation of the *Xanthomonas campestris* AvrBs2 protein to pepper plants. *PNAS*, **97**, 13324–13329.

Nagai, N., Kitauchi, F., Shimosaka, M., Okazaki, M. (1994): Cloning and sequencing of a full-length cDNA coding for phenylalanine ammonia lyase from tobacco cell culture. *Plant Physiol.*, **104,** 1091–1092.

Niyogi, K. K. (1999): Photoprotection revisited. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **50**, 391–417.

Nugent, J. M., Palmer, J. D. (1991): RNA-mediated transfer of the gene *coxII* from the mitochondrion to the nucleus during flowering plant evolution. *Cell*, **66,** 473–481.

Ochoa-Alejo, N., Gomez-Peralta, J. E. (1993): Activity of enzymes involved in capsaicin biosynthesis in callus tissue and fruits of chili pepper (*Capsicum annuum* L.). *J. Plant Physiol.*, **141**, 147–152.

Ohta, Y. (1963): Physiological and genetical studies on the pungency of *Capsicum*. IV. Secretory organ, receptacles and distribution of capsaicin in the *Capsicum* fruits. *Jap. J. Breeding*, **12,** 179–183.

Peterson, P. A. (1958): Cytoplasmically inherited male-sterility in *Capsicum*. *Am. Nat.*, **92**, 111–119.

Popovsky, S., Paran, I. (2000): Molecular genetics of the y locus in pepper: its relation to capsanthin-capsorubin synthase and to fruit color. *Theor. Appl. Genet.*, **101**, 86–89.

Pozueta-Romero, J., Houlne, G., Schantz, R. (1998): Identification of a short interspersed repetitive element in partially spliced transcripts of the bell pepper (*Capsicum annuum*) *PAP* gene: new evolutionary and regulatory aspects on plant tRNA-related SINEs. *Gene*, **214**, 51–58.

Pozueta-Romero, J., Klein, M., Houlne, G., Schantz, M. L., Meyer, B., Schantz, R. (1995): Characterization of a family of genes encoding a fruit-specific wound-stimulated protein of bell pepper (*Capsicum annuum*): identification of a new family of transposable elements. *Plant Mol. Biol.*, **28,** 1011–1025.

Pryor, T., Ellis, J. (1993): The genetic complexity of fungal resistance genes in plants. *Adv. Plant Path.*, **10**, 281–305.

Reinhammar, B. (1970): Purification and properties of laccase and stellacyanin from *Rhus vernicifera*. *Biochim. Biophys. Acta*, **205,** 35–47.

Richter, T. E., Pryor, T., Bennetzen, J. L., Hubert, S. H. (1995): New rust resistance specifities associated with recombination in the *Rp1* complex in maize. *Genetics*, **141**, 373–381.

Rushton, P. J., Somssich, I. E. (1998): Transcriptional control of plant genes responsive to pathogens. *Curr. Opin. Plant Biol.*, **1,** 311–315.

Ryals, J. A., Neuenschwander, U. H., Willits, M. G., Molina, A., Steiner, H. Y., Hunt, M. D. (1996): Systemic acquired resistance. *Plant Cell*, **8**, 1809–1819.

Ryan, C. A. (1990): Proteinase inhibitors in plants: genes for improving defenses against insects and pathogens. *Annu. Rev. Phytopathol.*, **28**, 425–449.

Sadanandom, A., Piffanelli, P., Knott, T., Robinson, C., Sharpe, A., Lydiate, D., Murphy, D., Fairbairn, D. (1996): Identification of a peptide methionine sulphoxide reductase gene in an oleosin promoter from *Brassica napus*. *Plant J.*, **10,** 235–242.

Schwarz, Z., Jolly, S. O., Steinmetz, A. A. (1981): Overlapping divergent genes in maize chloroplast chromosome and *in vitro* transcription of the gene for $tRNA^{HIS}$. *PNAS*, **78,** 3423–3428.

Shin, R., Kim, M. J., Paek, K. H. (2003a): The CaTin1 (*Capsicum annuum* TMV-induced clone 1) and CaTin1-2 genes are linked head-to-head and share a bidirectional promoter. *Plant Cell Physiol.*, **44,** 549–554.

Shin, R., Lee, G. J., Park, C. J., Kim, T. Y., You, J. S., Nam, Y. W., Paek, K. H. (2001): Isolation of pepper mRNAs differentially expressed during the hypersensitive response to tobacco mosaic virus and characterization of a proteinase inhibitor gene. *Plant Sci.,* **161,** 727–737.

Shin, R., Park, C. J., An, J. M., Paek, K. H. (2003b): A novel TMV-induced hot pepper cell wall protein gene (CaTin2) is associated with virus-specific hypersensitive response pathway. *Plant Mol. Biol.*, **51,** 687–701.

Shinozaki, K., Yamaguchi-Shinozaki, K. (1997): Gene expression and signal transduction in water-stress response. *Plant Physiol.*, **115**, 327–334.

Song, W. Y., Pi, L. Y., Wang, G. L., Gardner, J., Holsten, T., Ronald, P. C. (1997): Evolution of the rice *Xa21* disease resistance gene family. *Plant Cell.*, **9**, 1279–1287.

Sukrasno, N., Yeoman, M. M. (1993): Phenylpropanoid metabolism during growth and development of *Capsicum frutescens* fruits. *Phytochemistry*, **32**, 839–844.

Suzuki, T., Fujiwake, H., Iwai, K. (1980): Intracellular localization of capsaicin and its analogue, capsaicinoid, in *Capsicum* fruit. 1. Microscopic investigation of the structure of the placenta of *Capsicum annuum* var. *annuum* cv. Karayatsubusa. *Plant Cell Physiol.*, **21**, 839–853.

Tai, T. H., Dahlbeck, D., Clark, E. T., Gajiwala, P., Pasion, R., Whalen, M. C., Stall, R. E., Staskawicz, B. J. (1999): Expression of the Bs2 pepper gene confers resistance to bacterial spot disease in tomato. *PNAS*, **96**, 14153–14158.

Thorup, T. A., Tanyolac, B., Livingstone, K. D., Popovsky, S., Paran, I., Jahn, M. (2000): Candidate gene analysis of organ pigmentation loci in the *Solanaceae*. *Proc. Natl. Acad. Sci. USA*., **97**, 11192–11197.

Vishnevetsky, M., Ovadis, M., Vainstein, A. (1999): Carotenoid sequestration in plants: the role of carotenoid-associated proteins. *Trends Plant Sci.,* **4**, 232–235.

Vishnevetsky, M., Ovadis, M., Zuker, A., Vainstein, A. (1998): Molecular mechanisms underlying carotenogenesis in the chromoplast: multilevel regulation of carotenoid-associated genes. *Plant J.,* **20**, 423–431.

Wagner, S., Breiteneder, H. (2002): The latex-fruit syndrome. *Biochem Soc. Trans*., **30,** 935–940.

Ward, E. R., Uknes, S. J., Williams, S. C., Dincher, S. S., Wiederhold, D. L., Alexander, D. C., Ahl-Goy, P., Metraux, J., Ryals, J. A. (1991): Coordinate gene activity in response to agents that induce systemic acquired resistance. *Plant Cell*, **3**, 1085–1094.

Waterhouse, P. M., Smith, N. A., Wang, M. B. (1999): Virus resistance and gene silencing: killing the messenger. *Trends Plant Sci.*, **4,** 452–457.

Willerroider, M., Fuchs, H., Ballmer-Weber, B. K., Focke, M., Susani, M., Thalhamer, J., Ferreira, F., Wuthrich, B., Scheiner, O., Breiteneder, H., Hoffmann-Sommergruber, K. (2003): Cloning and molecular and immunological characterisation of two new food allergens, cap a 2 and Lyc e 1, profilins from bell pepper (*Capsicum annuum*) and tomato (*Lycopersicon esculentum*). *Int. Arch. Allergy Immunol.,* **131,** 245–255.

Yang, Y., Shah, J., Klessig, D. F. (1997): Signal perception and transduction in plant defense responses. *Genes Dev.,* **11**, 1621–1639.

Zavala-Páramo, G., Chávez-Moctezuma, M. P., Garcia-Pineda, E., Yin, S., Chappell, J., Lozoya-Gloria, E. (2000): Isolation of an elicitor-stimulated 5-*epi*-aristolochene synthase gene (g*PEAS*1) from chili pepper (*Capsicum annuum*). *Physiol. Plantarum*, **110**, 410–418.

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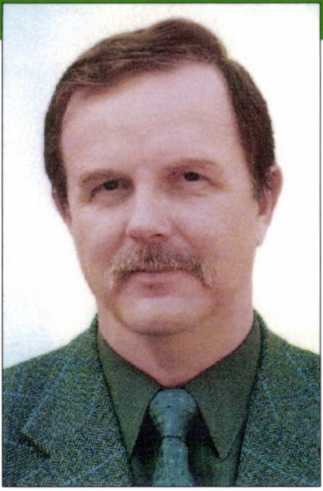

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ORIGINAL PAPERS

Acta Agronomica Hungarica, 54(3), pp. 271–279 (2006)
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QTL MAPPING AND GENETIC ANALYSIS OF INHIBITORY EFFECT OF LYSINE ON POST-GERMINATION GROWTH AND SEEDLING ESTABLISHMENT OF MAIZE

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Radicle elongation that allows rhizosphere colonization was used as a marker of seedling establishment. The goal of the present work was to determine the genetic basis of the effect of the aspartate family pathway, in particular lysine, on the radicle elongation of maize seedlings. For this purpose a population derived from an advanced backcross between a flint European line F2 and a highland tropical line F334 as the donor parent, was used for mapping QTLs related to the effect of lysine on radicle elongation. The parental lines showed contrasting germination efficiency and root elongation under both control conditions (imbibition on water at 20°C) and on medium supplied with lysine. Two QTLs for radicle elongation under control conditions were located on chromosome 2 (136 cM) near the marker bnlg1721 and on chromosome 5 (146 cM) near the marker umc1792. These QTLs explained 9.4% and 10.5%, respectively, of the phenotypic variability for radicle elongation. When germination was carried out on medium containing 5 mM lysine, three QTLs for radicle elongation were located on chromosome 7 (90 cM) near the marker umc1112, on chromosome 10 (42 cM) near the marker bnlg1037 and on chromosome 10 (68 cM) near the marker umc1053; these QTLs explained 12.0%, 12.3% and 12.4%, respectively, of the phenotypic variability for radicle elongation. Irrespective of the germination and post-germination medium, favourable alleles for all detected QTLs were associated with parental line F334.

Key words: lysine, *Zea mays*, QTL, post-germination growth, seedling establishment

Introduction

Discovering genes involved in germination efficiency and, more importantly, seedling establishment, and the mechanisms that allow the regulation of these processes is of primary scientific and agronomic interest. With the advent of molecular marker technologies, which allow dense genetic maps to be constructed from a single progeny, it became possible to dissect the

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genetic and molecular bases of complex (agronomic and physiological) traits by making an inventory of the loci (QTLs) involved in their phenotypic variation, to determine the mapping position of these QTLs and to estimate their effects. The 'effect' of a QTL may be quantified as the proportion of variance accounted for by the QTL, or, for a biallelic QTL, as the additive effect which corresponds to the half-difference of the homozygous mean values (Limami and De Vienne, 2001).

Several major QTLs related to agronomic traits in maize (*Zea mays*) (Limami et al., 2002) and rice (*Oryza sativa*) (Fujino et al., 2004) were found, some of which were cloned in *Zea mays* (Doebley et al., 1997), tomatoes (*Lycopersicon esculentem*) (Frary et al., 2000; Fridman et al., 2000; Liu et al., 2002) and *Oryza sativa* (Yano et al., 2000).

An approach combining molecular physiology and quantitative genetics using a 140 RIL population and parental lines (Io/F2) was undertaken to study the involvement of the nitrogen metabolism in germination and the early stages of development in maize (Limani et al., 2002). Among three QTLs for germination efficiency two were located in the vicinity of the *Akh1* (bifunctional Asp-kinase-homo-Ser dehydrogenase-1) locus on chromosome 4 and the *Ask2* (monofunctional Asp-kinase-2) and *Akh2* (bifunctional Asp-kinase-homo-Ser dehydrogenase-2) loci on chromosome 2. These genes encode isoforms of Asp kinase, the enzyme that catalyses the first step in the biosynthetic pathway of essential amino acids (Lys, Met and Thr). Therefore, these three Asp-kinase isogenes appeared to be suitable candidates to explain the phenotypic variation in germination performance and post-germination growth, suggesting the direct or indirect involvement of essential amino acids in the control of seedling establishment. Expression studies on these genes in the parental lines showed that the major difference concerned genes encoding bifunctional enzymes, particularly *Akh2*. ^{15}N-Asp labelling showed differences in the *in vivo* Asp-kinase activities between slow- and fast-germinating genotypes (F2/Io). The Asp flux into the Thr/Met branch was higher in Io than in F2, while the latter exhibited a higher flux of Asp into the lysine branch. Physiological results, together with the higher *Akh2* expression in Io, suggested that bifunctional enzyme activity favourable to Thr/Met was higher in Io than in F2 and that the monofunctional pathway was boosted in F2 because of lower competition by the bifunctional pathway, allowing for higher flux of Asp into the lysine branch (Anzala et al., 2006). Based on these studies, a hypothesis was put forward to explain how differences between genotypes in terms of speed of germination and post-germination growth were related to the essential amino acid metabolism, i.e. germination and post-germination growth might be partially inhibited in the slow-germinating genotypes due to the combined effects of a limitation in Met and Thr availability and the inhibitory effect of lysine on seedling growth. Lysine has been proposed as a good candidate to play a role in the control of growth and development (Zhu and Galili, 2004). Lysine accumulation in transgenic *Arabidopsis thaliana* due to the overexpression of dihydrodipicolinate synthase (DHPS) and a defective catabolism due to the inhibition of lysine ketoreductase (LKR) by the RNAi strategy exerted a negative physiological effect responsible for the inhibition of seedling growth (Zhu and Galili, 2004). The Asp family pathway received a great deal of attention from several researchers seeking to increase the content of nutritionally important amino acids, particularly lysine, in crop grains. It appeared that an increase in the lysine content by the genetic manipulation of its synthetic pathway failed because its content is controlled by both synthesis and catabolism (Cattoir-Reynaerts et al., 1981; Vauterin and Jacobs, 1994; Zhu and Galili, 2004), probably because lysine is not only a protein building block but plays a developmental role (Anzala et al., 2006).

The goal of the present work was to determine the genetic basis of the effect of lysine on germination and post-germination growth in maize. For this purpose a population derived from an advanced backcross between a flint European line F2 and a highland tropical line F334 as the donor parent, was used for mapping QTLs related to the effect of lysine on seedling establishment. The parental lines showed contrasting germination efficiency and root elongation under control conditions (imbibition on water) and in lysine-supplied medium. The expression of genes encoding four isoforms of aspartate kinase has been measured by q-RT-PCR in fast (Io and F334) and slow (F2) germinating maize genotypes during germination and post-germination growth, and the effect of exogenous lysine on seedling establishment, as quantified by the root elongation of Io, F334 and F2 and the advanced-backcross population, has been determined.

Materials and methods

Plant material

Three lines were chosen on the basis of their contrasting germination efficiency: an Iodent late line (I_o), an early French flint line (F2) and a Mexican highland line (F334).

A population of 125 maize (*Zea mays*) BC_2S_3 descents was used for the QTL experiment. This population was an advanced backcross. The F_1 generation, obtained by crossing F2 (early line) and F334, was backcrossed twice with F2. Then the plants were self-pollinated three times to obtain the generation used for genetic mapping. Phenotyping was done on seed bulks obtained by two additional self-pollinations.

Germination conditions

For the Asp-kinase (AK) experiment three maize genotypes, Io, F334 and F2, were germinated in Petri dishes (diameter 9 cm) on paper (Whatman, Clifton NJ) soaked with deionized water in a growth chamber at 20°C. Three replicates of 40 grains per Petri dish were used for the germination test (T50) and gene expression analysis. At various stages in the germination of each line (0, 24, 52, 74, 96 hours), 30 embryos of each replicate were pooled, frozen in liquid N_2 and stored at –80°C for further q-RT-PCR analysis.

For QTL detection a total of 125 BC_2S_3 descents and the parental lines (F2 and F334) were germinated in Petri dishes (diameter 9 cm) on Whatman paper soaked with deionised water or 5 mM lysine and maintained in a growth chamber at 20°C. Each seed was placed in an identical position in order to standardize germination efficiency. The radicle length was measured after 124 h of imbibition.

AK expression by q-RT-PCR

Total RNA was extracted from the frozen embryos as described by Verwoerd et al. (1989). q-RT-PCR reactions were performed as indicated by Anzala et al. (2006).

QTL detection

With the genetic map and the genotypic data as a basis, Grafgen software (Servin et al., 2002) was used to calculate for each individual the probability of the presence of each possible genotype (homozygous F2, homozygous F334 and heterozygous) along the 10 chromosomes, at intervals of 2 cM. Based on these probabilities, the F334 donor allele dose for each 2 cM interval was calculated. QTLs were detected by linear regression for each 2 cM interval with the PROC GLM procedure of the SAS software (version 8.1). LOD values were calculated from the F values as proposed by Knott and Haley (1992) and Lander and Botstein (1989). The LOD threshold was determined with 500 permutations. The LOD thresholds were determined for alpha-risks of 5%, 10% and 25%, corresponding to values of 2.82, 2.54 and 2.05, respectively. The confidence interval for each QTL position was determined by the LOD drop-off 1 unit method.

Results and discussion

Before launching the QTL analysis of the lysine effect on maize seedling establishment, the parental lines of the advanced backcross population, F334 and F2, were characterized. For this purpose the polymorphism of the parental lines was determined in terms of germination efficiency and root elongation either under standard conditions or on medium supplied with lysine. The expression of Asp kinase isogenes was quantified in the parental lines as a marker of polymorphism in the biosynthetic pathway of essential amino acids (Anzala et al., 2006). The genotype Io, which has been well characterized in former studies (Limami et al., 2002; Anzala et al., 2006), was added to the present study as a case of a fast-germinating genotype to be compared with F334.

Characterization of germination and post-germination growth and expression of genes encoding monofunctional and bifunctional isoforms of Asp kinase in embryos of slow- and fast-germinating maize genotypes

The time course of germination and root elongation in F334, Io and F2 was determined at 20°C in darkness. Germination curves (Fig. 1) showed that F334 exhibited the same pattern of germination as Io with very close values of T_{50} (time at which 50% of seeds germinated): 54 h and 52 h for F334 and Io, respectively. As expected, the slow-germinating genotype F2 exhibited a higher T_{50} (74 h) than F334 and Io.

Seedling establishment depends essentially on radicle elongation, which provide seedlings with the capacity to colonize the rhizosphere and to efficiently absorb water and mineral nutrients. Radicle elongation was determined and the results showed that F334 exhibited faster root growth (39.6 mm/h) under standard conditions than F2 (34 mm/h) and Io (30.5 mm/h). This showed that the cross between F334 and F2 is more suitable for studying QTLs for radicle elongation than the cross between Io and F2.

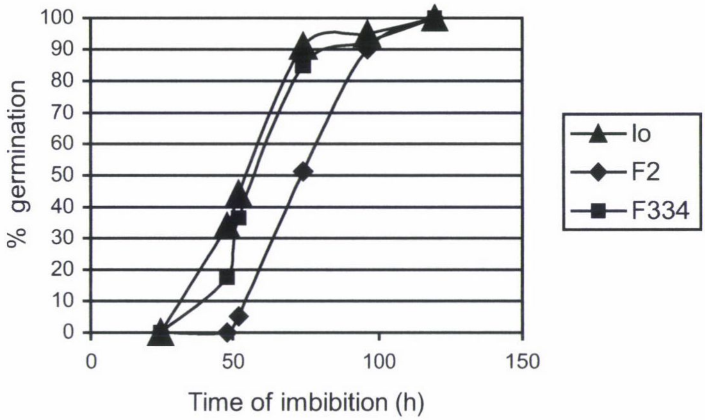

Fig. 1. Germination efficiency of three maize lines, Io, F334, and F2, determined as the percentage of germinated seeds at various times after the beginning of imbibition at 20°C

The expression pattern of Asp kinase isogenes was examined by real-time quantitative RT-PCR in embryos of F334, Io and F2 throughout germination and post-germination growth (Fig. 2). The expression of *Ask2*, *Akh2* and *Akh1* increased throughout germination and post-germination growth in the three genotypes. The expression of *Ask2*, *Akh2* and *Akh1* seemed to be developmentally regulated, whereas *Ask1* showed constitutive expression at a fairly constant level throughout germination and the post-germination growth period. Interestingly, the expression of Asp kinase isogenes was higher in the fast-germinating genotypes F334 and Io than in the slow-germinating genotype F2. This genetic variability concerned the four Asp kinase genes, indicating that the alleles of the genotypes F334 and Io conferred a higher level of expression than those of the genotype F2, resulting in polymorphism at the level of gene expression. The most marked polymorphism in terms of gene expression was observed for *Akh2*, the expression of which was markedly low in the slow-germinating genotype F2. After 52 h of imbibition, which corresponded to T_{50} for Io and almost T_{50} for F334, the expression of *Akh2* was roughly 3 and 6 times higher in Io and F334, respectively, than in F2.

Former works published by Wang and Larkins (2001), Wang et al. (2001) and Anzala et al. (2006) showed that differences in the expression of Asp kinase isogenes were accompanied by differences in the enzymatic activities of Asp kinase isoforms and in the free essential amino acid content. Considering the similarity of the pattern of expression of Asp kinase isogenes in both fast-germinating genotypes, it is suggested that the Asp flux might be higher into the threonine/methionine branch than into the lysine branch in the genotype F334, as previously shown in the genotype Io, and as a consequence the germination and seedling establishment of F334 is not submitted to the inhibitory effect of lysine, as shown in the genotype F2.

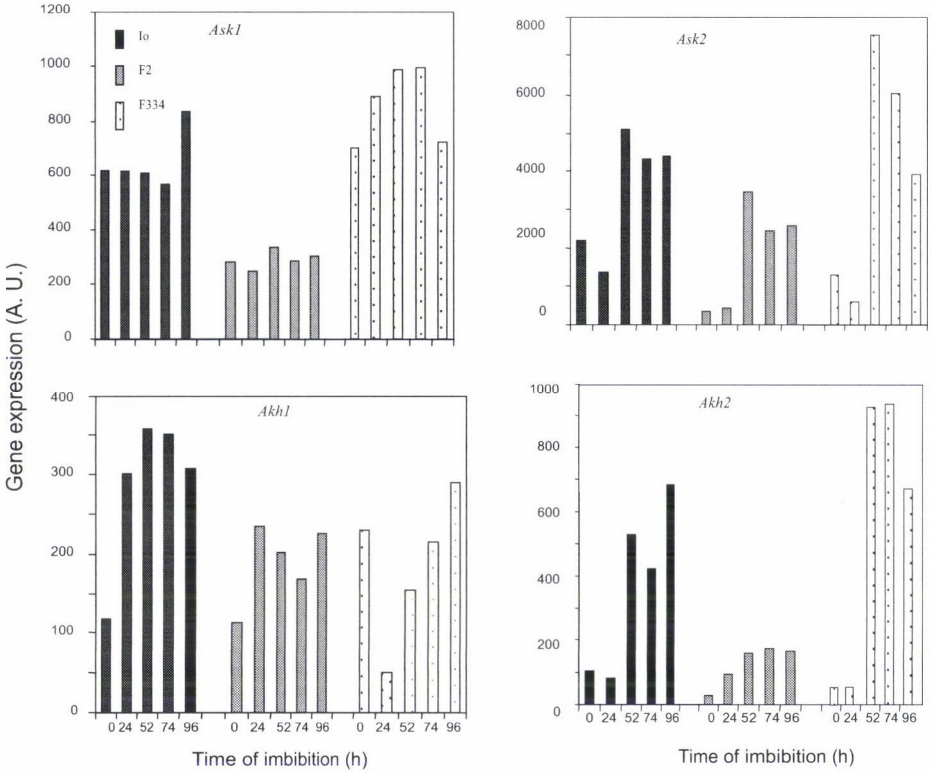

Fig. 2. Expression of aspartate kinase, *Ask1, Ask2, Akh1* and *Akh2,* throughout germination in maize. Q-RT-PCR was performed on total RNA extracted from the embryo axis at different times during the imbibition of three lines, Io, F2 and F334, germinated at 20°C

Effect of lysine treatment on the germination and post-germination growth of three homozygous maize genotypes Io, F334 and F2

In order to challenge the hypothesis of the effect of lysine on the early development and seedling establishment of maize, the germination and post-germination growth of genotypes Io, F334 and F2 on 5 mM lysine were compared to that on 5 mM methionine (osmotic control) and on demineralized water (control) (Fig. 3). The main outcome of this experiment was that the inhibitory effect of lysine on seed germination and seedling growth was exerted in both fast- and slow-germinating genotypes, and that this effect is not an osmotic effect, but is specific and physiologically relevant, since it has not been mimicked by methionine. The observed inhibitory effect of lysine on maize root elongation and consequently on seedling establishment supports the hypothesis of a signalling role, allowing lysine to exert a negative physiological effect responsible for the inhibition of *Arabidopsis thaliana* seedling growth (Zhu and Galili, 2004).

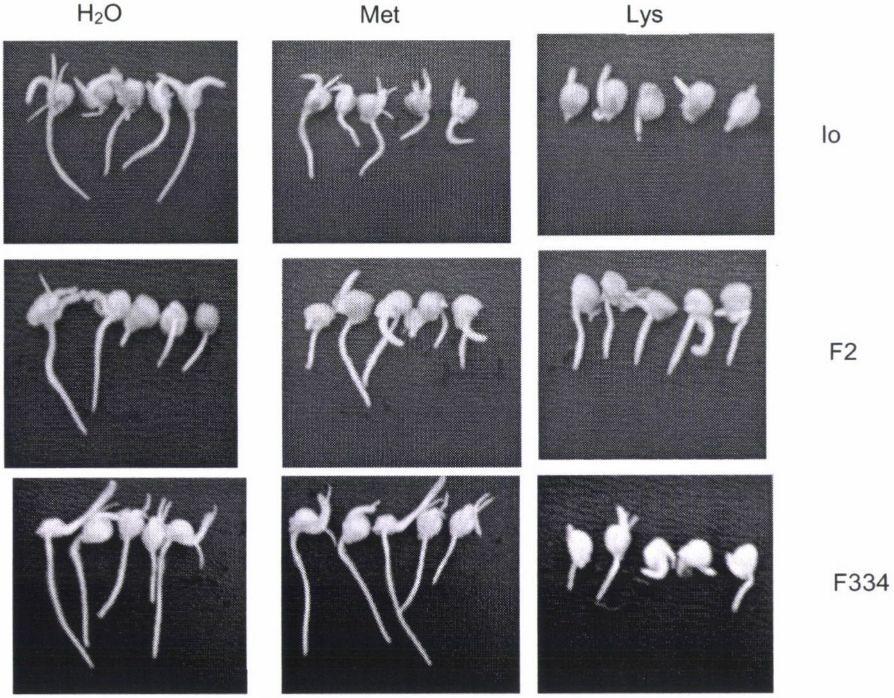

Fig. 3. Effect of methionine and lysine on radicle elongation. Picture of seeds after 124 h of imbibition

Mapping of QTLs related to root elongation under control conditions and on lysine-containing medium

A population derived from an advanced backcross between a flint European line F2 and a highland tropical line F334 as the donor parent, was used for mapping QTLs related to radicle elongation under control conditions (water) and on a medium containing 5 mM lysine. The results will facilitate the determination of the genetic basis of radicle elongation and the effect of lysine on this trait (Fig. 4). For this purpose the 125 lines of the advanced backcross population were germinated at 20°C in darkness on demineralized water (control) and on 5 mM lysine. The radicle length was measured for the individual lines and the speed of radicle growth was determined.

Two QTLs for radicle elongation under control conditions were located on chromosome 2 (136 cM) near the marker bnlg1721 and on chromosome 5 (146 cM) near the marker umc1792. These QTLs explained 9.4% and 10.5%, respectively, of the phenotypic variability for radicle elongation. When germination was carried out on medium containing 5 mM lysine, three QTLs for radicle elongation were located on chromosome 7 (90 cM) near the marker umc1112, on chromosome 10 (42 cM) near the marker bnlg1037 and on chromosome 10 (68 cM) near the marker umc1053. These QTLs explained 12.0%, 12.3% and 12.4%, respectively, of the phenotypic variability for radicle elongation.

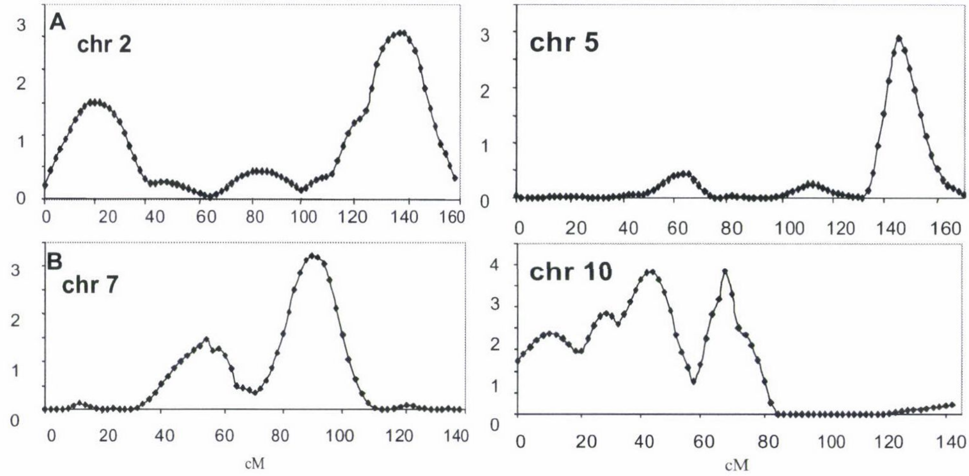

Fig. 4. QTL detection associated with radicle elongation. A) Under standard conditions. B) In medium containing 5 mM lysine. LOD threshold > 2.05

Irrespective of the medium of germination and post-germination growth, the favourable allele for all the QTLs was that originating from the parental line F334. Accordingly, the positive additive effect of the favourable allele was quantified for each QTL and it was found that the allele from F334 contributed to an increase in the speed of radicle growth with a mean value of 8.5 mm/h under control conditions and a mean value of 4.4 mm/h on lysine-containing medium. The results are in agreement with the fact that radicle growth was faster in F334 than in F2 and that lysine exerted an inhibitory effect on radicle growth.

Conclusions

In this study it was shown that three QTLs on chromosomes 2 and 5 (Fig. 4) were related to 'radicle elongation', indicating that this trait is controlled by genes or a cluster of genes located in these regions of the maize genome. Interestingly, when germination and post-germination occurred on lysine-containing medium three different QTLs on chromosomes 7 and 10 were related to 'radicle elongation'. The latter result supports the hypothesis that the aspartate family pathway may control radicle elongation and therefore seedling establishment via lysine production. It also indicates that the genes or cluster of genes involved in the control of radicle elongation, through which lysine exerts its inhibitory effect on radicle elongation, are different from those involved in the control of this trait under control conditions.

References

Anzala, F., Morere-Le Paven, M. C., Fournier, S., Rondeau, D., Limami, A. M. (2006): Physiological and molecular aspects of aspartate-derived amino acid metabolism during germination and post-germination growth in two maize genotypes differing in germination efficiency. *J. Exp. Bot.,* **57**, 645–653.

Cattoir-Reynaerts, A., Degryse, E., Negritiu, I., Jacobs, M. (1981): Effects of aspartate-derived amino acids on growth of barley and *Arabidopsis thaliana* plants and callus. *Z. Pflanzenphysiol.,* **1001**, 67–74.

Doebley, J., Stec, A., Hubbard, L. (1997): The evolution of apical dominance in maize. *Nature,* **386**, 485–488.

Frary, A., Nesbitt, T. C., Grandillo, S., Knaap, E., Cong, B., Liu, J., Meller, J., Elber, R., Alpert, K. B., Tanksley, S. D. (2000): fw2.2: a quantitative trait locus key to the evolution of tomato fruit size. *Science,* **289**, 85–88.

Fridman, E., Pleban, T., Zamir, D. (2000): A recombination hotspot delimits a wild-species quantitative trait locus for tomato sugar content to 484 bp within an invertase gene. *Proc. Natl. Acad. Sci. USA,* **97**, 4718–4723.

Fujino, K., Sekiguchi, H., Sato, T., Kiuchi, H., Nonoue, Y., Takeuchi, Y., Ando, T., Lin, S. Y., Yano, M. (2004): Mapping of quantitative trait loci controlling low-temperature germinability in rice (*Oryza sativa* L.). *Theor. Appl. Genet.,* **108**, 794–799.

Knott, S. A., Haley, C. S. (1992): Maximum likelihood mapping of quantitative trait loci using full-sib families. *Genetics,* **132**, 1211–1222.

Lander, E. S,, Botstein, D. (1989): Mapping mendelian factors underlying quantitative traits using RFLP linkage maps. *Genetics,* **121**, 185–199.

Limami, A. M., Rouillon, C., Glevarec, G., Gallais, A., Hirel, B. (2002): Genetic and physiological analysis of germination efficiency in maize in relation to nitrogen metabolism reveals the importance of cytosolic glutamine synthetase. *Plant Physiol.,* **130**, 1860–1870.

Limami, M., De Vienne, D. (2001): Natural genetic variability in nitrogen metabolis*m*. In: Lea, P. J., Morot-Gaudry, J. F. (eds.), *Plant Nitrogen*. Springer-Verlag, Heidelberg, pp. 368–397.

Liu, J., Van Eck, J., Cong, B., Tanksley, S. D. (2002): A new class of regulatory genes underlying the cause of pear-shaped tomato fruit. *Proc. Natl. Acad. Sci. USA,* **99**, 13302–13306.

Servin, B., Dillmann, C., Decoux, G., Hospital, F. (2002): MDM: A program to compute fully informative genotype frequencies in complex breeding schemes. *J. Hered.,* **93**, 227–228.

Vauterin, M., Jacobs, M. (1994): Isolation of a poplar and an *Arabidopsis thaliana* dihydrodipicolinate synthase cDNA clone. *Plant Mol. Bol.,* **25**, 545–550.

Verwoerd, T. C., Dekker, B. M., Hoekema, A. (1989): A small-scale procedure for the rapid isolation of plant RNAs. *Nucleic Acids Res.,* **17**, 2362.

Wang, X., Larkins, B. A. (2001): Genetic analysis of amino acid accumulation in opaque-2 maize endosperm. *Plant Physiol.,* **125**, 1766–1777.

Wang, X., Stumpf, D. K., Larkins, B. A. (2001): Aspartate kinase 2. A candidate gene of a quantitative trait locus influencing free amino acid content in maize endosperm. *Plant Physiol.,* **125**, 1778–1787.

Yano, M., Katayose, Y., Ashikari, M., Yamanouchi, U., Monna, L., Fuse, T., Baba, T., Yamamoto, K., Umehara, Y., Nagamura, Y., Sasaki, T. (2000): Hd1, a major photoperiod sensitivity quantitative trait locus in rice, is closely related to the *Arabidopsis* flowering time gene CONSTANS. *Plant Cell,* **12**, 2473–2484.

Zhu, X., Galili, G. (2004): Lysine metabolism is concurrently regulated by synthesis and catabolism in both reproductive and vegetative tissues. *Plant Physiol.,* **135**, 129–136.

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GENETIC AND EPIGENETIC REGULATION OF MALE FERTILITY RESTORATION IN THE 9E, A4 AND M35 CMS-INDUCING CYTOPLASMS OF SORGHUM

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The use of new CMS-inducing cytoplasms significantly increases the genetic variability of cultivated sorghum hybrids. The aim of the research was to study the inheritance of fertility restoration in new types of male-sterile cytoplasms (A4, 9E, M35) and to explore the reasons for its instability. Genetic analysis revealed that the restoration of male fertility was controlled by dominant genes, whose expression was strongly dependent on a sufficient level of water availability to plants during anther and pollen formation. An epigenetic mechanism for "switching on" fertility-restoring gene expression in F_1 hybrids is proposed, which is regulated by water availability to plants. This "active" state of fertility-restoring genes is stably expressed in self-pollinated progenies of restored hybrids, irrespective of their water availability conditions, but is not transmitted to testcross hybrids grown under arid conditions.

Key words: *Sorghum bicolor*, cytoplasmic male sterility, fertility restoration, epigenetic inheritance, drought

Introduction

An increase in the genetic variability of cultivated sorghum hybrids is one of the main problems in sorghum breeding. The use of new CMS-inducing cytoplasms could be favourable for creating diverse CMS lines and F_1 hybrids with desirable traits. However, difficulties in fertility restoration hamper the use of the majority of new types of CMS-inducing cytoplasms, whose common characteristic is the formation of large non-dehiscent anthers (A3, A4, 9E, M35) (Rao et al., 1984; Worstell et al., 1984; Torres-Cardona et al., 1990; Dahlberg and Madera-Torres, 1997; Pedersen et al., 2003). Accessions with fertility-restoring genes for these cytoplasms rarely occur and the gene-restorers themselves are not expressed stably in different hybrid combinations.

It was reported earlier that a few line-fertility restorers for the A4, 9E and M35 cytoplasms were found among early-maturing accessions adapted to the Volga Region of Russia (Elkonin et al., 1997a; b). Fertility restoration was consistently expressed in self-pollinated progenies of the restored F_1 hybrids but was not expressed at all or was poorly expressed in testcrosses of fertile plants from the F_2–F_3 generations with CMS lines with the same type of cytoplasm (Elkonin et al., 1998; Elkonin and Kozhemyakin, 2000). The aim of this research was to study the inheritance of fertility restoration and the effect of environmental factors, in particular water availability to plants, on the expression of fertility restoration.

Materials and methods

The line [9E] Tx398, containing CMS-inducing cytoplasm from the line 9E (Webster and Singh, 1964), and the line [A4] Tx398, containing cytoplasm from IS7920C (Worstell et al., 1984), were used as sources of the CMS-inducing cytoplasms 9E and A4, respectively. The source of the M35 cytoplasm was the Indian CMS line M-35-1A (Nagur and Menon, 1974). The seeds of these lines were generously provided by Dr. K. F. Schertz (Texas Agricultural Experimental Station, USA). These lines were used for obtaining new CMS lines by substitution backcrosses with the lines Milo-10, Ranneye-7 (denoted herein as Ran-7) and Pishchevoye-614 (P-614) (taken from the collection of the All-Russian Research Institute for Sorghum and Maize "Rossorgo", Saratov, Russia) and the line KVV-52 (developed by V. V. Kozhemyakin, Agricultural Research Institute for South-East Region, Saratov, Russia, from the hybrid population Rosinka-2/Norghum-165).

The male parental lines used in this study were selections from hybrid populations (*Sorghum bicolor*/sudangrass) obtained in our lab by V. V. Kozhemyakin (KVV-112, KVV-58, KVV-97), and the cultivars Perspectivnoye-1 (Pers-1) and Yubileinaya-20 (Yu-20, sudangrass), which were obtained from the "Rossorgo" collection.

All the CMS lines, the paternal lines, the F_1, F_2 and BC hybrids, and the testcross hybrids were grown in experimental fields located at the Agricultural Research Institute for South-East Region (Saratov, Russia) in 4–5 m rows.

The first panicle of each plant was bagged before anthesis. The panicles were carefully inspected 2–3 days after anthesis at the panicle base and were then selected for crosses. Plants which had no fertile anthers and pollen in the bags and which had 'fresh' stigmas on the whole panicle were considered 'male sterile' and were used for hand-pollination.

The level of male fertility was estimated at maturity by the percentage seed set. Depending on the percentage seed set the panicles were classified as sterile (s) (0% seed setting or 1–2 seeds), partially sterile (ps) (1–40%; usually on no more than the basal 1/3 of the panicle), partially fertile (pf) (40–75%; usually 2/3 of the panicle) and fertile (f) (> 75%).

To evaluate the influence of environmental conditions on the expression of fertility restoration the total precipitation was analysed (1) from planting to 50% anthesis, (2) three weeks before 50% anthesis (anther and pollen formation) and (3) prior to anthesis (microgametogenesis and pollen maturation). The midday temperature during these periods was also recorded.

To study the influence of the water availability to plants on fertility restoration, plants from three populations with the 9E, A4 and M35 cytoplasms were grown under irrigation during the period of anther and pollen formation. Two waterings, each with 25 l/m^2, were carried out. The first irrigation was at the beginning of the boot stage, which corresponded to the stage of anther initiation; the second irrigation was performed 7 days afterwards and before panicle emergence from the boot, at the stage of microsporogenesis completion and the formation of vacuolate pollen grains. Flowering data were recorded 6–8 days after the last watering. Control plants were grown in adjacent plots. All the experiments were replicated. In 2005, some F_2 and BC_1 hybrid combinations with 9E cytoplasm were grown additionally in a separate plot, which was covered by polyester film (only during rainy periods) for 3 weeks before anthesis to prevent the irrigation of the plants by rainfall.

The χ^2-test was used to determine the fit of observed ratios of sterile and fertile plants to the expected segregation ratio. The dependence of male fertility upon the total rainfall during anther and pollen formation was evaluated by correlation analysis. The number of plants with different levels of male fertility in different experiments was also compared by the Fisher method using the F-criteria (Zaitsev, 1984).

Results and discussion

Data on the inheritance of male fertility restoration in different hybrid combinations in different seasons are summarized in Table 1. Careful analysis of these data suggests the strong dependence of male fertility in sorghum hybrids with the 9E and A4 cytoplasms on the level of water availability to plants during anther and pollen formation. When the same F_1 populations ([9E]Milo-10/KVV-263, [9E]T×398/Pers-1, [9E]Milo-10/Pers-1) were grown in different seasons with different rainfall levels, there was a significant difference in the number of plants with restored male fertility, the higher precipitation level causing a higher number of fertile and partially fertile plants.

The same F_2 population ([A4] T×398/Pers-1) grown in two seasons that differed in the amount of precipitation before anthesis demonstrated significant differences in segregation ratios of fertile and sterile plants. In 2003, a relatively wet season, the percentage of fertile and partially fertile plants was significantly higher (P=0.01) than in 1999, a droughty season (Table 1). In 2003, the segregation in the F_2 fitted well to a digenic 15:1 ratio for fertility, while in 1999 the number of sterile plants increased, bringing the observed segregation ratio closer to a monogenic 3:1 ratio (for the 15:1 ratio $\chi^2 = 20.404$ and was not significant).

A digenic segregation ratio was also observed in other hybrid populations with the A4 and M35 cytoplasms when grown in wet seasons, while in the majority of hybrid populations with the 9E cytoplasm the monogenic 3:1 ratio was observed. In these seasons, segregation in the BC_1 generations was 1:1 for hybrid combinations which had a monogenic 3:1 segregation ratio in the F_2 generation, and 3:1 for those which had a digenic 15:1 segregation ratio in the F_2, thus confirming segregation for one or two fertility-restoring genes, respectively.

In the M35 cytoplasm, a strong positive correlation was observed between percentage of fertile and partially fertile plants (with a seed set >40%) in total samples of the self-pollinated and testcross hybrids, and the total precipitation during the three weeks before anthesis (r=0.93; P=0.05) (Fig. 1). No such correlation was found between temperature and total precipitation and its effects on male fertility.

Table 1

Inheritance of male fertility restoration in the 9E, A4 and M35 CMS-inducing cytoplasms of sorghum

Hybrid combination	Gen.	Year	Precipitation (mm)[1]	No. of plants[2] f	pf	ps	s	Pr. segregation	χ^2	*P* value
[9E]T×398/KVV-112	F_1	1992	≈5[3]	4	–	–	–			
	F_2	1993	70.7	11	–	–	3	3f:1s	0.095	0.75–0.90
(KVV-263)	F_5	1996	25.2	26	–	–	–			
	F_6	2002	0	12	–	–	–			
[9E] Milo-10/KVV-263	F_1	2002	0	–	–	1	11			
		2003	22.1	9	5	5	3			
KVV-263/Milo-10	F_1	2002	0	–	–	–	6			
KVV-263/T×398	F_1	2002	0	–	–	–	11			
[9E]P-614/Pers-1	F_1	1996	25.2	3	6	9	7			
	F_2	1997	47.8	14	–	–	–			
	F_3	1998	33.6	18	–	–	–			
[9E] P-614/F_2	BC_1	1998	14.6	–	–	–	8			
[9E] Milo-10/F_3	F_1	1999	3.4	–	–	–	35			
[9E] T×398/Pers-1	F_1	1999	3.4	9	2	8	4			
		2000	56.5	19	–	–	–			
	F_2	2001	9.9	5	6	18	9	3(f+pf+ps):1s	0.035	0.75–0.90
[9E] P-614/F_1	F_1	2001	9.9	–	–	3	32			
[9E]Milo-10/Pers-1	F_1	2002	5.8	9	4	3	–			
		2003	22.1	12	–	–	–			
	F_2	2003	22.1	61	4	1	4	15(f+pf+ps):1s	0.034	0.75–0.90
[9E]Milo-10/F_1	BC_1	2003	22.1	10	–	1	3	3f:1(ps+s)	0.095	0.75–0.90
[9E]Milo-10/Yu-20*	F_1	2004	49.3	1	2	3	15			
	F_2	2004	49.3	30	5	9	17	3(f+pf+ps):1s	0.268	0.50–0.75
	BC_1	2004	49.3	4	7	6	18	1(f+pf+ps):1s	0.029	0.75–0.90
[A4]Milo-10/Yu-20*	F_1	2004	49.3	1	1	2	19			
	F_2	2004	49.3	22	8	12	10	3(f+pf+ps):1s	0.923	0.25–0.50
	BC_1	2004	49.3	2	2	3	9	1(f+pf+ps):1s	0.250	0.50–0.75
[A4]T×398/Pers-1	F_1	1998	14.6	4	2	7	–			
		2003	22.1	23	15	16	2			
[A4]T×398/Pers-1	F_2	1999	3.4	20	4	10	10	3(f+pf+ps):1s	0.121	0.75–0.50
		2003	22.1	34	10	6	3	15(f+pf+ps):1s	0.031	0.90–0.75
[A4]T×398/KVV-97	F_1	1992	≈5 [3]	2	–	–	2			
	F_2	1997	47.8	20	6	5	4	15(f+pf+ps):1s	1.602	0.25–0.10
M35-1A / KVV-97	F_1	1993	70.7	2	–	–	12			
	F_2	1994	26.2	31	16	9	5	15(f+pf+ps):1s	0.395	0.75–0.50
[M35]P-614/F_2	F_1	1997	47.8	3	3	19	19			

Gen.: generation; Pr. segregation: proposed segregation; *(sudan grass) [1]for two weeks before anthesis; [2]f = fertile; pf = partially fertile; ps = partially sterile; s = sterile; [3]grown in greenhouse

The link between water availability and restoration was also observed in experiments on the artificial irrigation of F_1 hybrids having M35, A4 and 9E cytoplasms (Table 2). In these experiments, a significant increase in the percentage of fertile and partially fertile plants in treatments irrigated during anther and pollen formation was found. The most striking effect of artificial irrigation was observed in the F_1 hybrid [9E]Ran-7/KVV-263. Surprisingly, significant differences were observed in hybrids with A4 cytoplasm not only in a dry season (2002) but also in a wet season (2003), when precipitation itself increased the percentage of fertile plants.

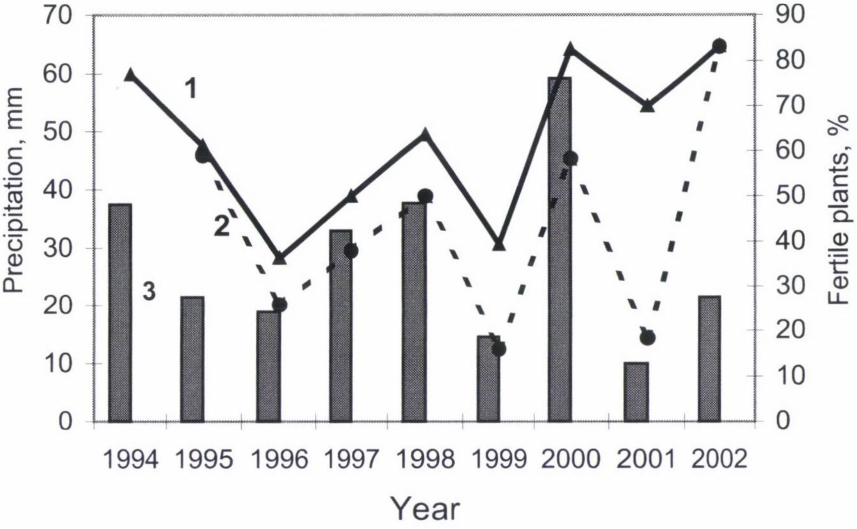

Fig. 1. Variability in the fertility of self-pollinated progeny of selections with M35 CMS-inducing cytoplasm (1), in testcrosses of fertile plants to the CMS line [M35] P-614 (2), and in the total precipitation 3 weeks prior to anthesis (3) from 1994 to 2002

Table 2

Influence of irrigation during anther and pollen formation (50 l/m^2) on the male fertility of F$_1$ hybrids with M35, A4 and 9E cytoplasms

Hybrid combination	Year	Natural precipitation[1]		Additional watering[2]	No. of plants	
		Before watering	During experiment		Total	Fertile and partially fertile[3], %
[M35]P-614/KVV45	2002	5.8	0	50	34	61.8**
				0	39	28.2
[A4]KVV-52/KVV-58	2002	5.8	0	50	72	40.4**
				0	91	17.0
[A4]T×398/Pers-1	2003	23.9	11.1	50	33	94.1*
				0	45	75.8
[9E]Ran-7/KVV-263	2005	16.0	7.5	50	11	72.7**
				0	15	0.0

[1] For 3 weeks before anthesis (mm): for 1 week before watering and for the next 2 weeks during the experiment; [2] Two waterings, each with 25 l/m^2: (1) at the stage of boot appearance, (2) 7 days later (before panicle emergence from the boot); flowering was recorded 6–8 days after the last watering; [3] with seed set >40%, means from two replications; [9E]Ran-7/KVV-263 was grown in only one replication; *, ** Significant at P=0.05 and P=0.01, respectively, according to the F-criterion.

The data in Table 1 also shed light on the reasons for the unusual inheritance pattern of fertility restoration in the 9E cytoplasm, namely, the stable inheritance of male fertility during self-pollination in the progeny of restored F$_1$ hybrids and the poor transmission through pollen in testcrosses of fertile hybrids to CMS lines with the same type of cytoplasm. For example, in the F_1 of crosses between the CMS lines [9E]T×398, [9E]Milo-10 and [9E]P-614 and the fertility restorers Pers-1 and KVV-112 either fertile plants or a combination of fertile, semi-fertile and sterile plants were observed. It was evident that the fertile F_1 hybrids should have the dominant fertility restorer genes. In the self-pollinated progenies of these fertile F_1 hybrids, fertile and partially fertile plants predominated, suggesting normal inheritance and a high level of expression of the dominant fertility-restorer genes. However, in testcrosses involving fertile plants from these progenies and CMS lines with the same cytoplasm ([9E] P-614 and [9E] Milo-10), only sterile plants or a mixture of sterile and a few partially sterile plants were observed (Table 1).

The most pronounced example of this was observed in the hybrids of a fertile line with the 9E cytoplasm, KVV-263, which was obtained by self-pollination from the fertile F_1 hybrid [9E]T×398/KVV-112 and therefore had dominant fertility-restoring genes. In the F_4 and F_5 generations this line produced only fertile plants and was hence homozygous for fertility-restoring genes. Nevertheless, this line produced only sterile and partially sterile F_1 hybrids in testcrosses with the CMS line [9E]Milo-10, obtained by backcrossing Milo-10 to [9E]T×398.

Careful analysis of the data revealed that the suppression of dominant fertility-restoring genes in the newly obtained F_1 hybrids was only observed when water stress occurred during pollen formation. For example, in 1996 and in 2002, under conditions of minimal water availability to plants during anthesis, all the F_1 hybrids of [9E] Milo-10/KVV-263 were sterile. However, in 2003, when water stress was not an issue, fertile and partially fertile plants predominated (Table 1). A high correlation (r=0.99; P=0.01) between the level of male fertility and the total precipitation during the 7 days before anthesis was also observed in 2003 (data not shown). Surprisingly, the line KVV-263 was completely fertile under both drought and wet conditions at anthesis.

The reciprocal hybrids, KVV-263/Milo-10 and KVV-263/T×398, which were obtained by hand-pollination of emasculated panicles of KVV-263 plants crossed to fertile maintainers of the CMS lines [9E]Milo-10 and [9E]T×398, were completely male-sterile in 2002 (Table 1). The male sterility of reciprocal F_1 hybrids excludes the hypothesis that cytoplasmic mutations occur in the sterile cytoplasm under the influence of nuclear genes, as a mechanism of male fertility restoration in the 9E cytoplasm, which might explain the stable inheritance of male fertility during self-pollination and its poor transmission in testcrosses, as takes place in CMS-*S* maize (Gabay-Laughnan et al., 1995), and in male-sterile common beans (He et al., 1995). These data suggest that under water-limiting conditions, the dominant fertility-restoring genes of KVV-263 were not expressed in the hybrid genome and the sterility of the F_1 hybrids was caused by the interaction of the sterile 9E cytoplasm of the KVV-263 line with nuclear genes from the sterility-maintainers. By contrast, in 2003, under conditions of sufficient moisture, the KVV-263/T×398 cross had both partially fertile and partially sterile plants, and no differences were seen between the direct ([9E]T×398/KVV-263) and reciprocal (KVV-263/T×398) combinations.

The sterility of both direct ([9E]Milo-10/KVV-263) and reciprocal (KVV-263/Milo-10) F_1 hybrids under drought conditions, as well as that of other testcross hybrids (Table 1), suggests that dominant fertility-restorer genes may become ineffective in a "new" hybrid background but remain effective in the genome of the parental lines or hybrids grown under the same environmental conditions (complete fertility of KVV-263). Evidently, this specific expression of fertility-restoring genes stems from the interaction of the parental alleles in the heterozygote, and, in this connection, is related to epigenetic phenomena, particularly the formation of alleles with stable expression, which are usually sensitive to environmental conditions. There may be an epigenetic mechanism that determines the expression level of fertility-restoring genes in the F_1 hybrids, which is regulated by water availability to the plants before anthesis. This "active" state of the fertility-restoring genes is stably inherited in subsequent self-pollinated progeny, but each time the expression level is established anew in the complex genome of the F_1 hybrids.

In order to obtain direct confirmation of the fact that the stable expression of fertility-restoring genes in self-pollinated progenies and their weak expression in testcrosses are due to the level of water availability to the plants before anthesis, the F_2 and BC_1 generations of two hybrid combinations with 9E cytoplasm were grown in parallel in 2005, in an irrigated plot and in an artificial dry treatment (see Material and Methods). In this experiment, a significant reduction in the percentage of fertile plants and an increase in partially sterile plants were observed in BC hybrids grown under dry conditions At the same time, no differences were recorded between the F_2 populations grown in the dry and irrigated plots (Table 3).

Table 3

Expression of male fertility in self-pollinated (F_2) progenies of sorghum hybrids with 9E cytoplasm and of testcrosses (BC_1) grown in parallel in irrigated plots and an artificial dry treatment

Hybrid combination	Generation	Irrigation/precipitation[1], >50 mm				Dry treatment 0 mm			
		f	pf	ps	s	f	pf	ps	s
[9E] Milo-10/Pers-1	F_2	23	1	1	3	8	2	–	–
[9E] Milo-10/([9E]Milo-10/Pers-1)	BC_1	9	–	–	–	–	–	3	10*
[9E] T×398/Pers-1	F_2	31	–	2	1	38	–	4	1
[9E] Milo-10/([9E]T×398/Pers-1)	BC_1	26	–	5	1	5	6	12*	2

[1] for 2 weeks before anthesis; *Significant at P=0.05, according to the F-criterion, in comparison with the irrigated plot

Thus, an interaction between genetic and epigenetic factors regulates male fertility restoration in the 9E, A4 and M35 CMS-inducing cytoplasms of sorghum.

In order to create reliable fertility-restoring lines, an attempt was made to reduce the high water requirement of fertility-restorer gene expression through the substitution of their genetic background. For this purpose, a line with fertility-restorer genes for the M35 cytoplasm (from the F_4 of M35-1A/KVV-97) was crossed with a sterile analogue ([M35] P-614) of the local line (Pishchevoye-614), which is characterized by intensive pollen formation under drought conditions. In 2002, which was characterized by severe drought during anther and pollen formation, the line KVV-45, having 80% fertile plants, was selected from the progeny of this hybrid. Its testcross populations with [M35]P-614 were also characterized by a high level of male fertility (up to 100%). In 2003, the line KVV-45 had a significantly higher frequency of fertile plants (100%) in comparison with the F_9 family (56%) derived from the original hybrid combination (M-35-1A/KVV-97) obtained by the constant selection of fertile plants without hybridization with [M35]P-614 ($P=0.001$).

Thus, by crossing a fertility-restorer gene donor with a local line and subsequent selection under drought conditions a reliable fertility restorer was created capable of restoring the male fertility of F_1 hybrids with the M35 cytoplasm under conditions of water stress.

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References

Dahlberg, J. A., Madera-Torres, P. (1997): Restorer reaction in A1 (AT×623), A2 (A2T×632), and A3 (A3SC 103) cytoplasms to selected accessions from the sudan sorghum collection. *Intern. Sorghum and Millet Newslett.*, **38**, 43–58.

Elkonin, L. A., Kozhemyakin, V. V. (2000): Cytoplasmic reversions as a possible mechanism of male-fertility restoration in the '9E' CMS-inducing cytoplasm of sorghum. *Intern. Sorghum and Millet Newslett.*, **41,** 30–31.

Elkonin, L. A., Kozhemyakin, V. V., Ishin, A. G. (1997a): Use of the novel CMS-inducing cytoplasms for development of early maturing sorghum lines with male sterility. *Doklady Russ. Acad. Agric. Sci.,* **2**, 7–9.

Elkonin, L. A., Kozhemyakin, V. V., Ishin, A. G. (1997b): Comparative analysis of restoration of male-fertility on the cytoplasmic male-sterile (CMS)-inducing cytoplasms A3 and M-35-1. *Intern. Sorghum and Millet Newslett.*, **38**, 29–30.

Elkonin, L. A., Kozhemyakin, V. V., Ishin, A. G. (1998): Nuclear-cytoplasmic interactions in restoration of male fertility in the '9E' and A4 CMS-inducing cytoplasms of sorghum. *Theor. Appl. Genet.*, **97**, 626–632.

Gabay-Laughnan, S., Zabala, G., Laughnan, J. R. (1995): *S*-type cytoplasmic male sterility in maize. pp. 395–452. In: Levings, III, C. S., Vasil, I. K. (eds.), *Molecular Biology of Plant Mitochondria.* Kluwer Academic Publ., Boston, Mass.

He, S., Lyznik, A., Mackenzie, S. (1995): Pollen fertility restoration by nuclear gene *Fr* in CMS bean: nuclear-directed alteration of a mitochondrial population. *Genetics*, **139**, 955–962.

Nagur, T., Menon, P. M. (1974): Characterization of different sterility-inducing cytoplasms in sorghum. *Sorghum Newslett.*, **17**, 18.

Pedersen, J. F., Marx, D. B., Funnell, D. L. (2003): Use of A3 cytoplasm to reduce risk of gene flow through sorghum pollen. *Crop Sci.*, **43**, 1506–1509.

Rao, N. G. P., Tripathi, D. P., Rana, B. S. (1984): Genetic analysis of cytoplasmic systems in sorghum. *Indian J. Genet. & Plant Breeding*, **44**, 480–496.

Torres-Cardona, S., Sotomayor-Rios, A., Quiles Belen, A., Schertz, K. F. (1990): Fertility restoration to A1, A2, and A3 cytoplasm systems of converted sorghum lines. *Texas Agr. Exp. Station*, **MP-1721**, pp. 1–11.

Webster, O. J., Singh, S. P. (1964): Breeding behavior and histological structure of a nondehiscent anther character in *Sorghum vulgare* Pers. *Crop Sci.*, **4**, 656–658.

Worstell, J. V., Kidd, H. J., Schertz, K. F. (1984): Relationships among male-sterility-inducing cytoplasms of sorghum. *Crop Sci.*, **24**, 186–189.

Zaitsev, G. N. (1984): *Mathematical Statistics in Experimental Botany*. Nauka, Moscow.

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GENETIC EVALUATION OF ROOT COMPLEXITY IN MAIZE

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Root architecture is strongly linked to plant survival under abiotic and biotic stress conditions. The objective of this study was to investigate the inheritance of the primary root system complexity in maize (*Zea mays* L.). For a total of 231 recombinant inbred lines (RIL) derived from the IBM (B73×Mo17) population multiple primary root systems were produced by applying a replicated alpha lattice experimental design. Digital images of each root system were taken at days four and eight after germination. For each root system image, the fractal dimension (FD) was computed. Significant differences between RILs were found in the FD calculated after four (FD1) and eight (FD2) days. For FD1 22 QTLs, for FD2 13, and for FD change over time (ΔFD) 12 QTLs were found on all ten maize chromosomes explaining between 24.6 and 46.8% of the phenotypic variation. Both parental inbreds contributed FD-increasing QTL alleles. FD1 and FD2 had five chromosomal BIN locations in common. Four unique QTLs were identified for the dynamics of root growth between days four and eight. Maize root mutants involved in root morphology were located in chromosomal BINs carrying QTLs for FD1 or FD2. This study demonstrated the usefulness of the IBM population as a maize community resource to investigate the genetic basis of root complexity in maize.

Key words: maize, QTL, primary root system, complexity, fractal dimension

Introduction

The development of a healthy root system is an important part of the overall plant development programme. Root branching and architecture are strongly linked to plant survival under abiotic (e.g. drought, flooding, nutrient deficiencies) and biotic (e.g. competition among plants, diseases, pests) stress conditions. A limited number of genetic studies is available relating maize root architecture and development with yield, root lodging, and tolerance to stresses under field conditions. This lack of in-depth knowledge is mainly due to the labour-intensive digging required to obtain root samples, the destructive nature of this procedure, and the highly heterogeneous root systems within and among

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different maize cultivars as a response to a complex soil matrix. Additionally, traditional measures such as root length and biomass do not provide an accurate quantification of root branching or complexity. In addition, root complexity and root development depend on genetic and environmental factors and their interactions (O'Toole and Bland, 1987).

The ability of plants to grow and produce seeds is governed by a functional and efficient root system. Complex root systems are characterized by a high number of branching points, having a higher probability of finding adequate resources by exploring a larger portion of the soil face than root systems with less complex root systems. The complexity of root systems can be determined by applying the mathematical fractal dimensions (FD). A key feature of fractals is self-similarity at varying scales, i.e. a small part of the structure resembles the whole structure. Fractals are dimensionless and fractal geometry allows for the description, study and analysis of complex shapes found in nature. In general, FD is more suitable to describe complex natural objects than standard Euclidian geometry (Mandelbrot, 1983).

Multiple studies demonstrated that various objects in nature are self-similar and can, therefore, be analysed employing fractal dimensions; examples include root systems (Tatsumi et al., 1989; Eghball et al., 1993; Lynch et al., 1993; Nielsen et al., 1997; Masi and Maranville, 1998; Oppelt et al., 2000; Walk et al., 2004), shoot systems and canopies of young trees (Morse et al., 1985; Foroutan-pour et al., 1999), seaweeds (Kubler and Dugeon, 1996), sponges (Abraham, 2001), neurons (Fernandez et al., 1994) and fungal mycelia (Mihail et al., 1995). The availability of adequate computer power and image analysis software packages allows for the application of FD to study root characteristics in more detail (Costa et al., 2001). Images for the study of FD have been previously acquired by video camera (Ottman and Timm, 1984; Cunningham et al., 1989) and optical scanner (Arsenault et al., 1995; Box, 1996; Kaspar and Ewing, 1997) as well as by photographic images (Tatsumi et al., 1989; Eghball et al., 1993; Masi and Maranville, 1998; Abraham, 2001), image transparencies (Nielsen et al., 1999) and SimRoot (Lynch et al., 1997). Despite this previous work on FDs there was to our knowledge no software package available that allows for an efficient large-scale screening of segregating QTL mapping populations.

The overall goal was to contribute to the scarce information about root morphology, complexity and development using images of primary root systems of maize. To achieve this goal a novel software package was developed for root image processing and complexity calculations. The specific objectives of this study were to (1) evaluate a large set of maize recombinant inbred lines (RIL) derived from the four times random-mated IBM (B73×Mo17) population for their fractal dimensions from root images and their variation over time, (2) determine quantitative genetic parameters for this characteristic, (3) map and characterize quantitative trait loci (QTL) affecting the complexity of primary root systems in maize, and (4) compare the root complexity QTLs with QTLs identified for other root morphology traits.

Materials and methods

Plant materials

A set of 240 recombinant inbred lines (RILs) selected from the IBM population were used. These RILs were developed by continuous selfing of randomly selected individuals from a four times random mated F_2 population developed from cross B73×Mo17 (Lee et al., 2002). Inbred B73 belongs to the Stiff Stalk Synthetic heterotic pool, whereas Mo17 is a non-Stiff Stalk inbred derived from the Lancaster pool.

Experimental design

The 240 RILs were subdivided into five sets. Each set comprised parental inbreds B73 and Mo17 as single entries and 48 RILs. The sets were evaluated in separate experiments. The experimental design for all experiments was a 10×5 alpha design with two replications.

Germination procedure

All seeds were surface sterilized with a commercial 6% Clorox® solution for 10 minutes. After this treatment, the seeds were washed three times with distilled and sterilized water. For each genotype, five seeds of the same genotype were placed in the upper third of a non-toxic germination paper. The embryo faced the bottom of the germination paper. The space between the seeds was maximized to prevent contact between different root systems. Each germination paper was moisturized with a Captan® (BAYER) 2.5 g l^{-1} solution, and afterwards rolled up vertically. Five rolled germination papers were placed vertically in one 2.5 l plastic bucket with 750 ml of distilled water plus 20 ml of Captan solution. All experiments were conducted in a germination chamber without illumination at 28ºC and 100% relative humidity. According to the field experiment terminology, each germination paper was regarded as a single plot and each bucket as an incomplete block.

Image processing

Images were taken four (Time 1) and eight days (Time 2) after germination with a Sony Cybershot 5.0 megapixels digital camera. The camera was mounted on a stand to standardize the image process. The background surface and the light in the room were taken into consideration to optimize the image quality. The images were acquired and saved in the JPEG format. A software package was developed to process the digital images of the maize primary root systems. The software package consisted of Matlab® (MATHWORKS) subroutines with the following six-step procedure.

Step 1: The RGB image was converted into a grey scale image.

Step 2: A square region of interest of the image containing the root was singled out to accommodate the requirements of the program used to calculate the FD.

Step 3: Thresholding was used to convert the grey-scale images into binary images.

Step 4: Histogram equalization was applied to further improve the image.

Step 5: The image was smoothed to remove noise by applying a median filter. Among all filters of equal size the median filter has an excellent noise reduction capability (Gonzalez and Woods, 2002).

Step 6: A Matlab subroutine was developed to measure FDs with a calculation program based on the box counting method as described by Mandelbrot (1983). According to this method the images were sequentially divided into grids of descending size and for each grid two values were recorded: N_s, the number of squares intersected by the image, and s, the side length of grid squares. FD is the regression coefficient describing the association between log (N_s) and log ($1/s$), which ranges from 1 to 2. It was expected that more complex embryonic root systems would have a larger FD. The variation of FD values between Time 1 (FD1) and Time 2 (FD2) was calculated as ΔFD=(FD1–FD2).

Statistical analysis

All data sets of the plant material evaluated were combined and an analysis of variance was performed applying the following model:

$$y = \mu + \alpha_i + \beta_{j(i)} + \delta_{k(ij)} + \gamma_l + \varepsilon_{(ijkl)m}$$

where y represents the phenotypic mean of a genotype, α_i is the effect of the i^{th} set, $\beta_{j(i)}$ is the effect of the j^{th} replication in the i^{th} set, $\delta_{k(ij}$ is the k^{th} block effect in the j^{th} replication of the i^{th} set, γ_l is the effect of the l^{th} genotype, and ε represents the residual error. All effects in the model were considered random. Estimates of the genotypic variance ($\sigma_g^{\;2}$), error variance (σ^2), and phenotypic variance ($\sigma_p^{\;2}$) and their standard errors were calculated as described by Searle (1971). Heritability estimates (h^2) for the RILs were calculated on an entry-mean basis as described by Hallauer and Miranda (1988). Phenotypic (r_p) correlation coefficients were calculated between traits by applying standard methods (Mode and Robinson, 1959). PLABSTAT (Utz, 1998) and SAS 9.1 (SAS Institute, 1996) software packages were used for all calculations.

QTL analysis

Linear unbiased predictors for 231 of 240 RILs were used in a single marker QTL analysis. Both molecular and phenotypic data were available only for this subset of RILs. The map for the random mated B73×Mo17 (IBM) population (Davis et al., 2001) is populated with > 1,000 RFLP and > 850 simple sequence repeat (SSR) markers. In this study, the genotypic data consisted of 1,167 markers that were evenly distributed across the maize genome. Composite interval mapping (CIM) was employed for QTL detection and the estimation of QTL effects. A LOD threshold of 3.6 was chosen for declaring a presumed QTL significant, ensuring a comparison-wise error rate of $P < 0.0003$ and an experiment-wise error rate of $P < 0.30$. Estimates of QTL positions were obtained where the LOD score reached its maximum in the region under consideration. All putative QTLs were examined for the presence of digenic epistatic effects. The portion of the phenotypic variance explained by all QTLs was determined by the adjusted coefficient of determination of regression ($R_{adj}^{\;2}$) fitting a model including all detected QTLs. All necessary calculations were performed with the PLABQTL (Utz and Melchinger, 1996) and SAS 9.1 (SAS Institute, 1996) software packages.

Results

Phenotypic evaluation

Histograms of the progeny means of 231 RILs are shown in Figure 1. Fractal dimensions determined four days after germination (FD1) varied between 1.084 and 1.157. Progeny means for FD determined eight days after germination (FD2) ranged from 1.080 to 1.202. Means of parental inbreds B73 and Mo17 were significantly ($P < 0.05$) different for FD2 but not for FD1. Heritability estimates were low for FD1 (0.32) and intermediate for FD2 (0.51) (Fig. 1). The phenotypic correlation of FD1 with FD2 was highly significant ($P < 0.01$) and of moderate size ($r_p = 0.70$).

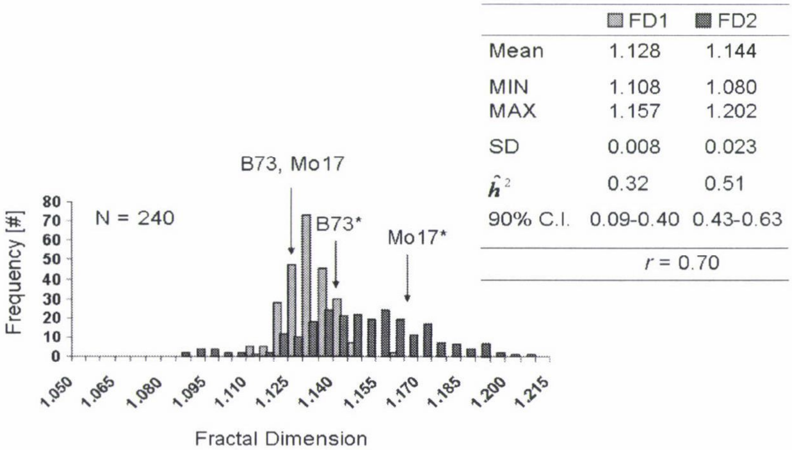
QTL analysis for root complexity

A total of 21 QTLs were found for FD1 on all chromosomes; these QTLs explained between 2.0 and 17.8% of $\hat{\sigma}_p^2$ (Table 1). Significant QTL interactions were identified between QTLs on chromosomes 1 (BIN 1.04) and 10 (BIN 10.07) as well as between QTLs on chromosomes 2 (BIN 2.04) and 7 (BIN 7.01/2) (see also Fig. 2). In a model fitting simultaneously all significant additive

QTL effects and additive×additive epistatic interactions, the two digenic interactions accounted for 3.5 and 3.7% of the phenotypic variation, respectively. In total, the simultaneous fit explained 46.8% of the phenotypic variation for FD1. Both parental inbreds contributed alleles that increased FD1.

A total of 13 QTLs were found for FD2. These QTLs were located on all chromosomes, except for chromosomes 6 and 9, explaining between 2.5 and 7.6% of $\hat{\sigma}_p^2$ (Table 3). A significant additive×additive epistatic interaction was found between BINs 5.03 and 7.00 (see also Fig. 2). In a model fitting all significant additive QTL effects and additive×additive epistatic interactions simultaneously, this digenic interaction accounted for 5.0% of the phenotypic variation. All QTLs explained 37.5% of the phenotypic variation for FD2. Both parental inbreds contributed alleles that increased FD2 values. Although the QTL in BIN 7.00 was detected to be significant (LOD < 3.6), the simultaneous fit showed a non-significant main effect.

As to ΔFD, 12 significant QTLs explaining between 1.8 and 10.2% of $\hat{\sigma}_p^2$ were found on chromosomes 1 (2 QTLs), 2, 4 (2 QTLs), 5, 7 (2 QTLs), 8 (2 QTLs) and 9 (2 QTLs) (Table 3). No significant digenic interactions were identified among these QTLs (Fig. 2). Nine of 12 marker intervals showed a negative genetic effect. In a model fitting all significant additive QTL effects simultaneously in one model, 24.6% of the phenotypic variation for ΔFD was accounted for. Parental inbred B73 contributed the alleles increasing ΔFD for nine out of 12 QTLs.



	▨ FD1	▩ FD2
Mean	1.128	1.144
MIN	1.108	1.080
MAX	1.157	1.202
SD	0.008	0.023
$\hat{h}^2$	0.32	0.51
90% C.I.	0.09-0.40	0.43-0.63
	$r = 0.70$	

Fig. 1. Histogram for fractal dimensions of primary root systems of 231 RILs derived from the cross B73×Mo17 after four (FD1) and eight (FD2) days of germination. Arrows indicate the means of parental lines B73 and Mo17. Stars indicate parental means for FD2

Table 1

Parameters associated with QTLs for FD of primary root systems measured four days after germination (FD1). Parameters were estimated from phenotypic data of 231 RILs derived from the cross B73×Mo17

BIN[†]	Pos. cM	Marker interval		*LOD*	Genetic effect[‡]	$R^2_{partial}$[§]	$R^2_{(total)adj}$
		Left	Right				
1.01	2.8	MO005	MO006	3.78	0.001*	2.0	
1.04[#]	*40.0*	*MO059*	*MO060*	*7.44*	*ns*		
1.07	68.2	MO103	MO104	4.04	0.001*	3.2	
1.09	82.0	MO127	MO128	8.79	–0.002**	5.5	
1.10	90.4	MO136	MO137	8.65	0.001**	4.5	
1.11	100.8	MO150	MO151	11.92	0.002**	13.3	
2.04	28.6	MO209	MO210	6.41	–0.001**	3.8	
3.06	45.4	MO372	MO373	7.62	0.002**	8.3	
4.09	63.2	MO527	MO528	5.95	0.002**	10.0	
5.03	24.8	MO583	MO584	7.93	–0.002**	8.6	
5.05	38.6	MO617	MO618	14.84	–0.003**	17.8	
6.04	20.8	MO697	MO698	5.75	0.001*	2.2	
6.05	26.8	MO707	MO708	5.01	–0.001**	4.0	
7.01/2	12.8	MO779	MO780	7.16	0.001**	3.6	
7.02	22.8	MO797	MO798	4.27	–0.001*	3.2	
8.01	4.6	MO874	MO875	4.67	–0.001*	2.8	
8.03	23.2	MO901	MO902	7.73	0.002**	11.5	
8.07	46.6	MO935	MO936	13.49	0.001**	5.1	
9.01	1.0	MO962	MO963	5.08	0.002**	10.1	
10.00	2.2	MO1075	MO1076	5.61	0.001**	5.4	
10.07	45.0	MO1157	MO1158	4.35	0.001*	3.2	
10.07	48.2	MO1160	MO1161	5.77	–0.001**	5.2	
		1/400	10/482		0.001**	3.5	
		2/286	7/128		–0.001**	3.7	46.8

[†]BIN locations are designated by an X.Y code, where X is the linkage group containing the BIN and Y is the location of the BIN within the linkage group (Gardiner et al., 1993). [‡]Additive genetic effects were estimated in a simultaneous fit using multiple regression.[§] R^2 = Proportion of phenotypic variance explained by the respective QTL. Total adjusted R^2 was calculated in a simultaneous fit using multiple regression. [#]QTL with LOD value larger than 3.5 but with non-significant genetic effect, as determined by a simultaneous fit using multiple regression

Common QTLs across traits

For FD1 and FD2, four common QTL regions were found (1.11, 5.03, 8.07 and 10.00). BINs 7.02 and 9.01 harboured QTLs for FD1 and ΔFD. Traits FD2 and ΔFD shared no common QTLs (Fig. 2).

Table 2

Parameters associated with QTLs for FD of primary root systems measured eight days after germination (FD2). Parameters were estimated from phenotypic data of 231 RILs derived from the cross B73×Mo17

BIN†	Pos. cM	Marker interval		*LOD*	Genetic effect‡	R^2_{partial}§	$R^2_{\text{(total)adj}}$
		Left	Right				
1.02	12.2	MO019	MO020	7.28	0.005**	7.6	
1.11	100.8	MO150	MO151	6.60	0.004**	4.6	
2.01	4.6	MO169	MO170	8.21	0.004**	5.8	
2.07	42.8	MO240	MO241	8.34	−0.004**	6.1	
3.04	21.2	MO319	MO320	8.3	−0.005**	6.2	
3.05	32.4	MO348	MO349	4.21	0.005**	6.8	
4.06	39.2	MO484	MO485	11.01	−0.003**	3.2	
4.11	74.4	MO542	MO543	4.77	−0.004**	4.7	
5.01	12.8	MO560	MO561	8.77	0.004**	3.8	
5.03	22.6	MO577	MO578	6.22	−0.004**	4.5	
7.00#	*4.8*	*MO766*	*MO767*	*5.09*	*ns*		
7.03	34.0	MO816	MO817	6.24	−0.003*	2.8	
8.07	46.4	MO935	MO936	4.64	0.003*	2.5	
10.00	2.2	MO1075	MO1076	7.34	0.004**	4.0	
		5/226	7/48		0.004**	5.0	37.5

†BIN locations are designated by an X.Y code, where X is the linkage group containing the BIN and Y is the location of the BIN within the linkage group (Gardiner et al., 1993). ‡Additive genetic effects were estimated in a simultaneous fit using multiple regression.§R^2 = Proportion of phenotypic variance explained by the respective QTL. Total adjusted R^2 was calculated in a simultaneous fit using multiple regression. #QTL with LOD value larger than 3.5 but non-significant genetic effect, as determined by a simultaneous fit using multiple regression.

Table 3

Parameters associated with QTLs for the change of FDs over time (ΔFD). Parameters were estimated from phenotypic data of 231 RILs derived from the cross B73×Mo17

BIN†	Pos. cM	Marker interval		*LOD*	Genetic effect‡	R^2_{partial}§	$R^2_{\text{(total)adj}}$
		Left	Right				
1.03	30.2	MO045	MO045	3.73	−0.007**	3.1	
1.06	60.6	MO096	MO097	3.78	−0.005*	1.8	
2.09	69.0	MO276	MO277	12.24	−0.009**	5.5	
4.01	0.8	MO423	MO424	3.97	0.007**	3.6	
4.05	29.6	MO470	MO471	3.62	−0.005*	2.5	
5.08	62.2	MO645	MO646	6.47	−0.013**	10.1	
7.02	16.8	MO786	MO787	8.81	0.014**	8.0	
7.02	18.0	MO790	MO791	6.40	−0.011**	5.1	
8.04	32.4	MO916	MO917	5.04	−0.013**	10.2	
8.05	36.6	MO924	MO925	6.72	0.010**	5.8	
9.01	1.8	MO964	MO965	8.81	−0.008**	4.6	
9.02	10.0	MO977	MO978	3.61	−0.006**	3.1	24.6

†BIN locations are designated by an X.Y code, where X is the linkage group containing the BIN and Y is the location of the BIN within the linkage group (Gardiner et al., 1993).‡Additive genetic effects were estimated in a simultaneous fit using multiple regression.§R^2 = Proportion of phenotypic variance explained by the respective QTL.Total adjusted R^2 was calculated in a simultaneous fit using multiple regression.#QTL with LOD value larger than 3.5 but non-significant genetic effect, as determined by a simultaneous fit using multiple regression.

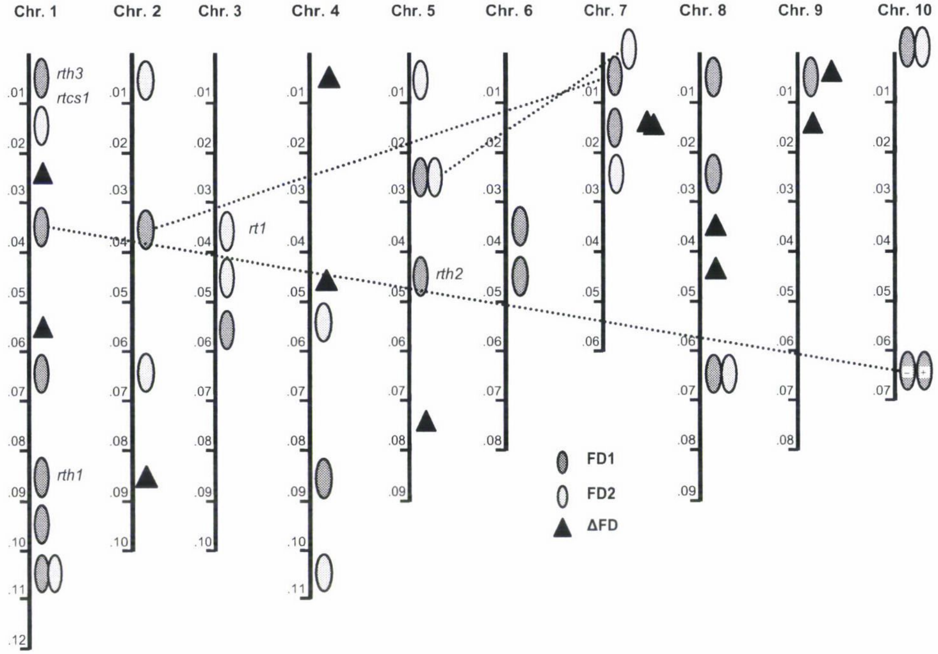


Fig. 2. Schematic linkage map reporting QTLs for FD of primary roots determined 4 and 8 days after germination from the cross B73×Mo17. Numbers to the left of the chromosome indicate the respective BIN. Chromosomal regions carrying QTLs for FD of primary roots determined 4 and 8 days after germination are represented by ovals and QTL locations for the change of FD over time are indicated by triangles; the dashed line indicates significant digenic additive×additive epistatic interactions; plus and minus signs in ovals describe signs of QTL effect

Discussion

The overall mean of FD for the primary root system of the parental inbreds B73 and Mo17 and the RILs increased over time. This observation is in agreement with the increased number and length of lateral and seminal roots (data not presented) found eight days after germination, which resulted in an increased root complexity. However, the difference between the overall population means for FD1 and FD2 were small. This finding could be explained by the short time interval between image acquisitions. However, based on a previous study (Fonseca, 2005) evaluating a diverse set of maize inbreds for primary root complexity, the four-day interval between image acquisition was sufficient for observing a significant increase in root length, width and branching. According to fractal geometry, a line has a dimension of one independently of its length. Therefore, if the system expands just in one direction without further branching the FD of a root system will not change. The analysis of the dynamic change of root complexity over time, as determined by ΔFD, in the IBM population revealed significant differences among RILs for their root development. RILs with negative ΔFD values were also observed, a finding that might indicate that after an initial complex development only the primary root continued to grow.

The significant genotypic variation for FD1 and FD2 observed among the IBM RILs is in agreement with findings in other species. Tatsumi et al. (1989) determined FD values for root systems of different plant species varying between 1.48 and 1.58. FD values of maize root systems ranged from 1.40 to 1.73 if the plants were subjected to nitrogen stress (Eghball et al., 1993). Estimates of FD for different sorghum [*Sorghum bicolor* (L.) Moench] genotypes varied between 1.44 and 1.89 and FD values for soybean roots [*Glycine max* (L.) Merr.] changed with increasing planting densities (low density: $1.30 < FD < 1.67$; high density: $1.15 < FD < 1.36$) (Foroutan-pour et al., 1999). Oppelt et al. (2000) reported FD values ranging between 1.17 and 1.66 for the fruit tree species *Strychnos spinosa*, *Vangueria infausta* and *Strychnos cocculaides* and for the shrub *Grewia flava*.

In this study, the complexity of the primary root system of maize in the two-dimensional space was determined by restricting the growth of the root system in germination paper rolls. In order to capture the complexity of adult maize root systems this approach must be extended into the three-dimensional space. One possible solution to this complex problem might be to count the number of root tips. Abraham (2001) studied the fractal branching of the sponge species *Raspailia inaequalis* and found a high correlation between the number of tips/end points and the FD values of the three-dimensional structure. For plant species no studies are yet available to confirm this relationship.

Applying composite interval mapping, a large number of QTLs ($N \geq 12$ QTL) were found for FD and its change over time. Even though these FD1, FD2 and ΔFD were significantly correlated with each other and the number of detected QTLs was large, only 7 out of 40 chromosomal BINs carrying QTLs were found to contain QTLs for at least two traits. If QTLs in adjacent BINs are also regarded as common, 12 chromosomal regions with multiple QTLs were identified. This moderate level of congruency could be accounted for by the small proportion of the phenotypic variation explained by the identified QTL, indicating that several additional QTLs with small effects remain to be detected. Possible ways to increase the power of QTL detection are to consider larger population sizes ($N > 500$) and to improve the accuracy of the phenotyping. Another possible explanation for the limited QTL overlap across traits is that FD values measured at different primary root development stages capture different sets of uncorrelated root complexity-defining characteristics. In agreement with this hypothesis, Fonseca (2005) reported only moderate to low associations between root characteristics determined 6 and 12 days after germination for a set of 45 diverse maize inbreds. The presence of different root growth patterns as described by Gao and Bohn (2004) might also explain the observed lack of QTL congruency.

Mutants affecting root morphology have been studied in *Arabidopsis*, soybeans (Kosslak et al., 1997) and tomato (Zobel, 1991). Also for maize, several mutants affecting the formation of root hairs (*rth1*, *rth2*, *rth3*), lateral roots (*lrt1*, *slr1*, *slr2*) and shoot-borne roots (*rtcs*, *rt1*) were found (Jenkins, 1930; Hetz et al., 1996; Hochholdinger et al., 2004). Evaluation of these mutants demonstrated that primary, lateral and adventitious root formation was partly controlled by different sets of genes. For six maize root mutants their chromosomal BIN location is known. It is worth noting that root mutants have been mapped to all the BINs that have been shown to carry QTL for FD1 or FD2 (Fig. 1) in this study. Following the hypothesis of Robertson (1985) it can be speculated that such mutants represent extreme alleles for the genes underlying the QTL identified in this study.

Transgressive segregation was observed for FD1, FD2 and ΔFD. In agreement with this observation, both parental inbreds contributed QTL alleles that increased FD. It is interesting to note that for ΔFD, 9 out of 12 QTLs with increasing effects were contributed by parent Mo17. This is in accordance with the significantly higher FD2 and ΔFD means found for parent Mo17 than for parent B73. Previous observations confirm significant growth rate differences for the primary root between parents Mo17 and B73 (Fonseca, 2005).

Tuberosa et al. (2002) summarized QTL results found in the literature for the primary root characteristics, including length and diameter, of maize plants grown in hydroponics. About half of the QTL locations identified in this study aligned with QTL locations reported for primary root length and diameter. However, new putative QTL locations were also identified due to the use of different parental germplasm and the application of new traits that take into account root complexity and its dynamics.

Conclusions

This study demonstrated that FD differences between RILs of the IBM population have a genetic basis. Applying a QTL mapping approach, it was possible to identify chromosomal regions in the maize genome carrying putative genes for root complexity. Some of these QTL regions were associated with root mutants providing logical candidate genes for root complexity. However, only limited information is available about the genes underlying these mutants. In addition, most QTLs were located in regions with no further information about possible candidate genes explaining the observed genotypic variation for root complexity.

This study also provides the technical basis for a systematic investigation of maize root complexity. All images used to determine fractal dimensions are available to investigate the usefulness of other complexity measures, such as entropy. Root complexity needs to be correlated with conventional root characteristics, such as root length, number of seminal roots, root angle, root hair density or root biomass. Within this project first ideas were developed to determine the complexity of adult root systems in all three dimensions. Consequently, the complexity of primary and secondary root systems needs to be associated with important agronomic traits, such as root lodging, drought tolerance, N-uptake efficiency, tolerance/resistance to insects and/or diseases. The root evaluation system deployed herein may help maize breeders to more efficiently screen maize germplasm for important root characteristics. In addition, knowledge about the genetic relationship between root complexity and agronomic traits could accelerate the development of improved maize cultivars.

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References

Abraham, E. R. (2001): The fractal branching of an arborescent sponge. *Marine Biol.,* **138**, 503–510.

Arsenault, J. L., Poulcur, S., Messier, C., Guay, R. (1995): WinRHIZO, a root-measuring system with a unique overlap correction method. *Hort Sci.*, **30**, 906.

Box, J. E. (1996): Modern methods for root investigations. pp. 193–237. In: Waisel, Y., Eshel, A., Kafkafi, U. (eds.), *Plant Roots: The Hidden Half.* Marcel Dekker, New York.

Costa, C., Dwyer, L. M., Hamel, C., Muamba, D. F., Wang, X. L., Nantais, L., Smith, D. L. (2001): Root contrast enhancement for measurement with optical scanner-based image analysis. *Can. J. Bot.*, **79**, 23–29.

Cunningham, M., Adams, M. B., Luxmoore, R. J., Post, W. M., DeAngelis, D. L. (1989): Quick estimates of root length, using a video image analyzer. *Can. J. For Res.,* **19**, 335–340.

Davis, G., Musket, T., Melia-Hancock, S., Duru, N., Sharopova, N., Schultz, L., McMullen, M., Sanchez, H., Schroeder, S., Garcia, A. A. (2001): The intermated B73 × Mo17 genetic map: A community resource. *Maize Genetics Conference Abstracts,* **43**, W15, P62.

Eghball, B., Settimi, J. R., Maranville, J. W., Parkhurst, A. M. (1993): Fractal analysis for morphological description of corn roots under nitrogen stress. *Agron. J.,* **85**, 287–289.

Eghball, B., Schepers, J. S., Negahban, M., Schlemmer, M. R. (2003): Spatial and temporal variability of soil nitrate and corn yield: multifractal analysis. *Agron. J.*, **95**, 339–346.

Fernandez, E., Eldred, W. D., Ammermuller, J., Block, A., von Bloh, W., Kolb, H. (1994): Complexity and scaling properties of amacrine, ganglion, horizontal, and bipolar cells in the turtle retina. *J. Comp. Neurol.*, **347**, 397–408.

Fonseca, M. L. (2005): Evaluation of root characteristics in maize and QTL mapping for the same root characteristics. Faculdade de Ciências da Universidade do Porto. Relatório de estágio.

Foroutan-pour, K., Dutilleul, P., Smith, D. L. (1999): Soybean canopy development as affected by population density and intercropping with corn: fractal analysis in comparison with other quantitative approaches. *Crop Sci.,* **39**, 1784–1791.

Gao, Y., Bohn, M. (2004): *Development of a bioassay to investigate maize* (*Zea mays* L.) – *Western corn rootworm* (*Diabrotica virgifera* LeConte) *interaction*. University of Illinois, ACES, RAP II Internship Research Paper.

Gardiner, J. M., Coe, E. H., Melia-Hancock, S., Hoisington, D. A., Chao, S. (1993): Development of a core RFLP map in maize using an immortalized F2-population. *Genetics*, **134**, 917–930.

Gonzalez, R. C., Woods, R. E. (2002): *Digital Image Processing*. 2nd Edition. Prentice Hall

Hallauer, A. R., Miranda, J. B. (1988): *Quantitative Genetics in Maize Breeding*. Iowa State University Press, Ames.

Hetz, W., Hochholdinger, F., Schwall, M. (1996): Isolation and characterization of rtcs, a maize mutant deficient in the formation of nodal roots. *Plant J.*, **10**, 845–857.

Hochholdinger, F., Woll., K., Sauer, M., Dembinsky, D. (2004): Genetic dissection of root formation in maize (*Zea mays*) reveals root-type specific development programmes. *Ann. Bot.*, **93**, 359–368.

Jenkins, M. T. (1930): Heritable characters of maize XXXIV- Rootless. *J. Hered.*, **21**, 79–80.

Kaspar, T. C., Ewing, R. P. (1997): ROOTEDGE: software for measuring root length from desktop scanner images. *Agron. J.*, **89**, 932–940.

Kosslak, R. M., Chamberlain, M. A., Palmer, R. G., Brown, B. A. (1997): Programmed cell death in the root cortex of soybean root necrosis mutants. *Plant J.*, **11**, 729–745.

Kubler, J. E., Dugeon, S. R. (1996): Temperature dependent changes in the complexity of form of *Chondrus crispus* fronds. *J. Exp. Mar. Biol. Ecol.*, **207**, 15–24.

Lee, M., Sharopova, N., Beavis, W. D., Grant, D., Katt, M., Blair, D., Hallauer, A. (2002): Expanding the genetic map of maize with the intermated B73 × Mo17 (IBM) population. *Plant Mol. Biol.*, **48**, 453–461.

Lynch, J., Johannes, J., Beem, V. (1993): Crop physiology and metabolism: growth and architecture of seedling roots of common bean genotypes. *Crop Sci.*, **33**, 1253–1257.

Lynch, J. P., Nielsen, K. L., Davis, R. D., Jablokow, A. G. (1997): SimRoot: Modelling and visualization of root systems. *Plant Soil*, **188**, 139–151.

Mandelbrot, B. B. (1983): *The Fractal Geometry of Nature*. Freeman, New York.

Masi, C. E. A., Maranville, J. W. (1998): Evaluation of sorghum root branching using fractals. *J. Agri. Sci., Cambridge*, **131**, 259–265.

Mihail, J. D., Obert, M., Bruhn, J. N., Taylor, S. J. (1995): Fractal geometry of diffuse mycelia and rhizomorphs of *Armillaria* species. *Mycological Res.*, **99**, 81–88.

Mode, C. J., Robinson, H. F. (1959): Pleiotropism and the genetic variance and covariance. *Biometrics*, Alexandria, **15,** pp. 518–537.

Morse, D. R., Lawton, J. H., Dodson, M. M. (1985): Fractal dimension of vegetation and the distribution of arthropod body lengths. *Nature*, **314**, 731–733.

Nielsen, K. L., Lynch, J. P., Weiss, H. N. (1997): Fractal geometry of bean root systems: correlation between spatial and fractal dimension. *Am. J. Bot.*, **84**, 26–33.

Nielsen, K. L., Miller, C. R., Beck, D., Lynch, J. P. (1999): Fractal geometry of root systems: field observations of contrasting genotypes of common bean (*Phaseolus vulgaris* L.) grown under different phosphorus regimes. *Plant and Soil*, **206**, 181–190.

O'Toole, J. C., Bland, W. L. (1987): Genotypic variation in crop plant root systems. *Adv. Agron.*, **41**, 91–145

Oppelt, A., Kurth, W., Dzierzon, H., Jentschke, G., Godbold, D. (2000): Structure and fractal dimensions of root systems of four co-occurring fruit tree species from Botswana. *Ann. For. Sci.*, **57**, 463–475.

Ottman, M. S., Timm, H. (1984): Measurement of viable plant roots with the image analysing computer. *Agron. J.*, **76**, 1018–1020.

Robertson, D. S. (1985): A possible technique for isolating genic DNA for quantitative traits in plants. *J. Theor. Biol.*, **117**, 1–10.

SAS Institute, Inc. (1996): *SAS/STAT user's guide*. Version 9.1. SAS Institute, Cary, N.C.

Searle, S. R. (1971): *Linear Models*. John Wiley & Sons, New York. 475 p.

Tatsumi, J., Yamauchi, A., Kono, Y. (1989): Fractal analysis of plant root systems. *Ann. Bot.*, **64**, 499–503.

Tuberosa, R., Salvi, S., Sanguineti, M. C., Landi, P., Maccaferri, M., Conti, S. (2002): Mapping QTLs regulating morpho-physiological traits and yield: Case studies, shortcomings and perspectives in drought-stressed maize. *Ann. Bot.*, **89**, 941–963.

Utz, H. F. (1998): *PLABSTAT. Ein Computerprogramm zur statistischen Analyse von pflanzenzüchterischen Experimenten.* Institute of Plant Breeding, Seed Science, and Population Genetics, University of Hohenheim, Stuttgart, Germany.

Utz, H. F., Melchinger, A. E. (1996): PLABQTL: A program for composite interval mapping of QTL. J. Quant. Trait. Loci, 2:1 (http://www.uni-hohenheim.de)

Walk, T. C., Van Erp, E., Lynch, J. P. (2004): Modelling applicability of fractal analysis to efficiency of soil exploration by roots. *Ann. Bot.*, **94**, 119–128.

Zobel, R. W. (1991): Genetic control of root systems. pp. 21–30. In: Waisel, Y., Eshel, A., Kafkafi, U. (eds.), *Plant Roots: The Hidden Half.* Marcel Dekker, New York.

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STUDIES ON POLYMORPHISM AND RELATED GROUPS IN MAIZE USING GENETIC MARKERS

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The analysis of polymorphism between 46 maize inbred lines with known genetic background and the classification of these lines in related groups was carried out by means of morphological, isoenzyme and genetic markers. The degree of relationship between the lines was determined using cluster analysis. Only a very limited extent of allele polymorphism could be detected in isoenzyme analyses. Nevertheless, on the basis of RAPD and SSR markers, all the lines could be distinguished from each other. Grouping lines into related groups it was found that, while the individual marker systems only partially reflected the actual relationships, a joint analysis of genetic markers and morphological data revealed a close correlation between the groups formed on the dendrogram and genetic backgrounds.

Key words: maize, polymorphism, isoenzyme, RAPD, SSR

Introduction

The CPVO (Community Plant Variety Office) guidelines elaborated for the registration and Plant Variety Protection (PVP) of maize varieties are based on the criteria of distinctness, uniformity and stability (DUS). However, a description based on morphological traits is often not sufficient to satisfy these conditions. One of the main fields where the detection of differences between varieties is required is the establishment of intellectual property rights, so it is essential to use techniques which allow the varieties to be distinguished accurately and reliably (Smith and Smith, 1989b). The rapid development of marker technologies has led to their widespread use in various fields of plant breeding, including maize breeding. Molecular markers have been used to facilitate the cataloguing of genetic stocks, to measure genetic variability and genetic distances, and to provide variety descriptions for identification and patenting purposes (Smith and Senior, 1999).

The CPVO regulations now make it compulsory to carry out starch gel electrophoresis to identify the isoenzyme patterns of maize varieties, based in most cases on the enzymes of the citrate and pentose phosphate cycles (Bourgoin-Greneche and Lallemand, 1993). As the genetic background of enzyme-coding genes, their location on the chromosomes and the possible alleles are all well known, a study of these genes can be used in most cases to check all three criteria included in the DUS tests (Goodman and Stuber, 1983; Stuber et al., 1988; Smith and Smith, 1992).

The grouping of maize varieties into related groups is of great importance in breeding, since genetic distances between parental lines is a basic criterion for heterosis breeding. Molecular techniques which, directly or indirectly, map the genetic background of individual plants are indispensable for this work (Smith et al., 1997; Pejic et al., 1998).

In the course of the present studies, in addition to morphological description and isoenzyme analysis, 46 maize inbred lines with known genetic background were analysed using PCR-based techniques involving RAPD and gene-linked microsatellite (SSR) markers. The aim of the work was to determine how efficiently and in what combinations these marker systems could be used to demonstrate genetic polymorphism between maize inbred lines and to map relationships between the lines.

Materials and methods

Plant material

46 maize inbred lines with known pedigrees, chosen from major germplasm groups, were studied: Lancaster, Iodent, Iowa Stiff Stalk Synthetic (ISSS), Mindszentpuszta Yellow Dent (MYD) and OP Lacaune. Additional lines, which cannot be classified in any of the above-mentioned groups but whose origin is precisely known, were also included: Argentinian flints, early Canadian lines, lines related to W 117 and derivatives of Co 125. Due to the large number of lines, two lines, derived either from each other or from common parents, were chosen from each related group for the analysis of degrees of relationship.

Morphological description

The lines were sown, one to a row, in small-plot experiments set up in a random block design with four replications at two locations in Hungary in two years. The scored traits were determined from the mean of 10 plants per plot, as described by the European Union CPVO-TP/2/2 protocol (Anonymous, 2001) and Smith and Smith (1989a).

Isoenzyme analysis

MDH (maleic acid dehydrogenase, 6 loci), IDH (isocitrate dehydrogenase, 2 loci), PGD (6-phosphogluconate dehydrogenase, 2 loci), PGI (phosphoglucose isomerase, 1 locus), PGM (phosphoglucomutase, 2 loci), ACP (acidic phosphatase, 1 locus) and ADH (alcohol dehydrogenase, 1 locus) were examined by starch gel electrophoresis (Goodman and Stuber, 1983; Stuber et al., 1988).

Genetic analyses

Genomic DNA was isolated from seedlings of maize inbred lines according to Dweikat et al. (1994) and Gyulai et al. (2000). The PCR reaction was carried out as described by Weining and Langridge (1991). The maize inbred lines were tested with 20 RAPD and 20 SSR primers.

Statistical evaluation of the results

The data were evaluated using the SPSS (Norusis, 1993) cluster analysis programme on the basis of standardised morphological data, and a presence-absence data matrix for the alleles of PCR fragments and enzyme loci exhibiting polymorphism (Gyulai et al., 2000).

The Dice similarity indexes were calculated by comparing pairs of lines according to the formula $GS = 2N_{ij}/(N_i + N_j)$, where GS represents the genetic similarity between inbred lines i and j, N_{ij} is the number of fragments common to i and j and N_i and N_j are the total number of fragments detected in lines i and j, respectively.

The PIC (polymorphism index content) values expressing the degree of polymorphism were calculated using the equation $1 - \Sigma f_i^2$, where f_i represents the frequency of the i^{th} allele at the given locus (Smith et al., 1997).

Results and discussion

Polymorphism analysis on the basis of morphological descriptions

According to the results of analysis of variance, the environment had no significant effect on certain traits, such as the anthocyanine coloration of the spikelet husk, the shape of the ear, the kernel type, the colour of the kernel crown, the number of kernel rows, or the anthocyanine coloration of the cob. The anthocyanine coloration of the anther, the spikelet ring and the stigma, the degree of fertilisation of the ear and the width of the kernels exhibited only a slight dependence on the year (significant at the 10% level of probability). The environment had a great influence on the kernel colour, where the data were significantly different at the 1% level of probability. The remaining traits were significantly affected by the environment ($p = 0.1\%$).

On the basis of the CPVO-TP/2/2 (2001) application form, two lines, *Mo 17 Mv* and its isogenic derivative, *Mo 17 wx*, exhibited such strong similarity that they could not be distinguished on the basis of the fundamental traits listed on the form.

Polymorphism analysis on the basis of isoenzyme patterns

Thirteen of the 15 enzyme loci examined exhibited polymorphism on the basis of isoenzyme patterns. Of the 35 alleles possible at these loci, 29 were found in the tested lines, equivalent to a mean value of 2.2 alleles/locus.

When determining polymorphism on the basis of isoenzyme patterns, the 46 lines were found to form 29 different gel electrophoresis groups, indicating that some of the lines could not be distinguished from each other. In the majority of cases, this similarity was due to the relationship between the lines and could be attributed to their having the same genetic background. In a few cases, however, the similarity between the isoenzyme patterns could not be explained on the basis of pedigree.

On the basis of the isoenzyme pattern 18 lines exhibited distinct gel electrophoresis patterns, manifested not so much in the appearance of unique alleles, as in distinct combinations of alleles at the polymorphic loci.

A similar picture was obtained in the case of Dice similarity indexes. The highest value was obtained for lines exhibiting identical enzyme patterns (Dice index = 1), which for most lines could be explained by the genetic background. The lowest value was obtained between *Mv L2* and *F7* (0.38), which corresponds well with the origin of the lines, which are not related to each other at all.

The two Lancaster lines, *Mo 17 Mv* and its isogenic variant, *Mo 17 wx*, could not be distinguished from each other on the basis of isoenzyme pattern either.

The PIC (polymorphic index content) values expressing the degree of polymorphism ranged from 0.04 to 0.55 for the isoenzyme loci, with a mean value of 0.27. Among the polymorphic enzyme loci the lowest values were found for loci *Mdh3*, *Mdh5* and *Pgm1* (PIC = 0.04). The greatest amount of information with respect to the presence of polymorphism was provided by loci *Pgm2* and *Acp1*, but even these only had PIC values of 0.55. The extremely low mean value and the fact that even the highest value was not very high indicate that the efficiency of isoenzyme analysis in demonstrating polymorphism is extremely limited.

Polymorphism analysis using PCR-based markers (RAPD, SSR)

Analysis involving data from RAPD and microsatellites (SSR) was effective in detecting polymorphism among all the maize inbred lines tested.

Of the 20 RAPD primers tested, *OP/AB 2*, *OP/AB 3*, *OP/AB 6*, *OP/AB 9* and *OP/AB 13* did not give clear results even after several replications.

Three primers (*OP/AB 9*, *OP/AB 15* and *OP/AB 19*) exhibited a 100% monomorphic pattern. Other RAPD primers (*OP/AB/4*, *OP/AB7*, *OP/AB/10*, *OP/AB/14*, *OP/AB 18* and *OP/AB 20*) exhibited extremely selective polymorphism between the lines. Finally, 12 RAPD primers were chosen, with a total of 93 fragments, of which 54 (averaging 4.5 fragments/primer) gave a reliable polymorphic pattern in all the replications (Fig. 1).

The Dice similarity index was high (0.8–0.9) in several related groups (e.g. ISSS, Lancaster, the early Canadian lines), which can be explained by the origin of the lines. The lowest Dice indexes calculated during the RAPD analysis were obtained between lines which, in the light of their genetic background, were known to be unrelated to each other. The polymorphism between the two lines exhibiting the greatest similarity, *CM 105* and *CM 108* (Dice similarity coefficient: 0.91), was due to differences in two fragments on each of three primers (*OP/AB 4*, *OP/AB 10* and *OP/AB 14*).

In the analysis of SSR markers, primer pairs linked to the genes *wx*, *wx1*, *cko1* and *adh2* did not give PCR patterns suitable for evaluation, as the pattern was 100% monomorphic for *zp1* and *p1* and almost 98% monomorphic for *mir2*.

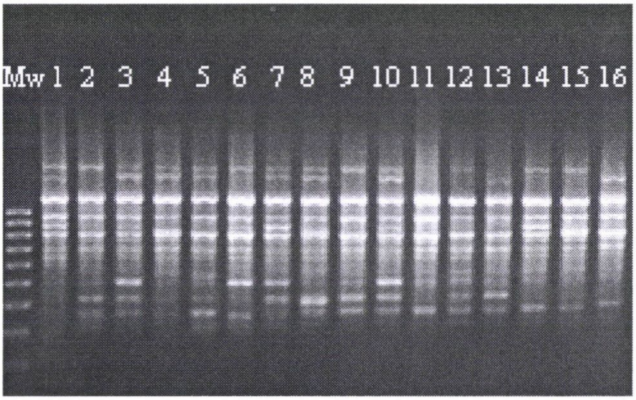

Fig. 1. Analysis of polymorphism in maize lines (1–16) using the OP/AB-20 RAPD primer (Mw: molecular weight marker)

Although two of the SSR loci linked to the genes *ohp2* and *mlg3* exhibited polymorphism, the patterns could not be identified because the differences between the fragments were extremely small, amounting to only a few base pairs. Several SSR loci linked to the *nac1*, *mgs1*, *pl1*, *gln4* and *op2* genes gave extremely selective polymorphism between the lines (Fig. 2).

The data matrix was finally compiled on the basis of 71 polymorphic fragments from 11 primer pairs, representing 6.4 fragments/primer.

The selective polymorphism characteristic of the SSR markers was reflected in the Dice indexes, which had much lower values than in the case of isoenzyme patterns or RAPD analysis. High values were rarely found, and even these were lower than the highest values recorded for the other two methods. This indicates that the SSR markers provide an extremely selective analysis of polymorphism even with a low number of primers. The greatest similarity was found between lines *Mv L8* and *Mv L10* (Dice similarity coefficient: 0.85), the polymorphism of which was caused by two fragments from each of two microsatellite primer pairs linked to the genes *pl1* and *sus1*.

An analysis of genetic markers enabled polymorphism to be detected for all the lines, which is particularly noteworthy in the case of isogenic lines (a line and its *wx* variant, or a line and its fertility restoring, *rf*, variant).

The two Lancaster lines, *Mo 17 Mv* and *Mo 17 wx*, which did not exhibit polymorphism on the basis of either morphological description or isoenzyme pattern, could be distinguished by means of both SSR and RAPD marker analysis.

The PIC (polymorphic index content) values expressing the degree of polymorphism were in line with the above observations.

The PIC values obtained for RAPD and SSR markers were the highest, ranging from 0.20–0.91 for RAPD, with an average of 0.61, and from 0.54–0.90 for SSR, with an average of 0.73. This indicates that the RAPD and SSR markers gave a reliable picture of polymorphism even with a relatively low number of primers.

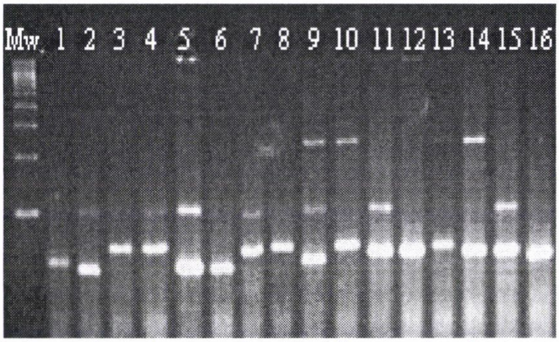

Fig. 2. Analysis of polymorphism in maize lines (1–16) using the o2 microsatellite primer pair (Mw: molecular weight marker)

Analysis of degrees of relationship

To analyse degrees of relationship two lines, derived either from each other or from common parents, were chosen from each related group.

On the basis of morphological descriptions, the groups formed on the dendrogram did not reflect the real relationships for all the lines (Fig. 3).

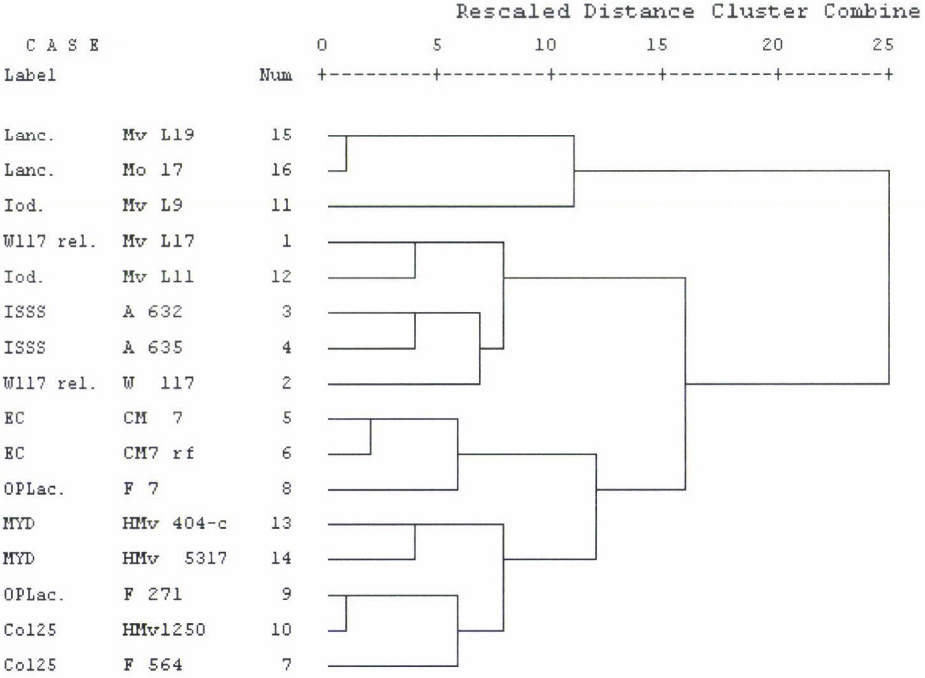

Fig. 3. Related groups on the basis of morphological traits
(ISSS: Iowa Stiff Stalk Synthetic; EC: Early Canadian; W117 rel.: lines related to W 117; Iod.: Iodent; Lanc.: Lancaster; OPLac.: OP Lacaune; MYD: Mindszentpuszta Yellow Dent; Co125: Co 125 derivatives)

Lines developed from the Lancaster (*Mo 17 Mv*, *Mv L19*), ISSS (*A 632*, *A 635*), Early Canadian (*CM 7*, *CM 7 rf*) and Mindszentpuszta Yellow Dent (*HMv 404-c*, *HMv5317*) varieties and derivatives of *Co 125* (*F 271*, *HMv 1250*) were found in the same cluster, as expected from the relationship between them, while lines related to Iodent (*Mv L9*, *Mv L11*), OP Lacaune (*F 564*, *F 7*) and *W 117* (*W 117*, *Mv L17*) were associated with members of other groups.

A similar picture was obtained when genetic markers were analysed, among which the SSR patterns reflected real relationships to the least extent. Naturally, this was due to the low number of primer pairs, not to the inefficiency of the method. As the SSR markers are to be found in the hypervariable regions of the chromosomes, a far larger number of primer pairs are required than in the analysis of isoenzymes, which act as structural genes. When the genetic markers were subjected to combined analysis, all the lines with the exception of Iodent derivatives (*Mv L9*, *Mv L11*) were classified in the group expected from their origin (Fig. 4).

The genetic background was reflected most accurately by the dendrogram produced by the combined analysis of genetic markers, combined with morphological data, which classified the related groups according to their genetic background (Fig. 5). The clusters indicating related groups could be clearly distinguished from each other, and only contained line pairs which were related to each other.

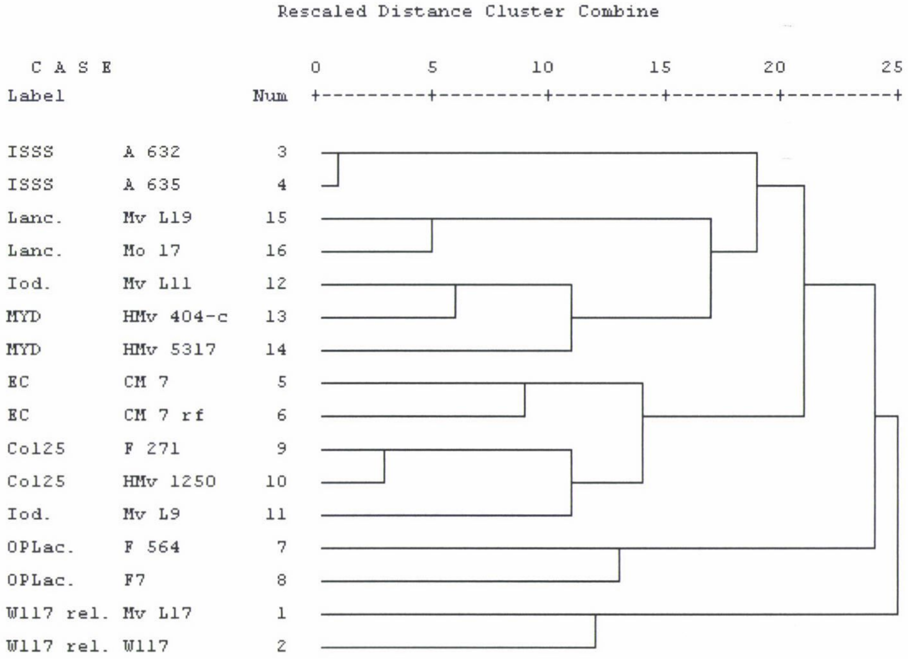

Fig. 4. Related groups on the basis of patterns given by genetic markers (isoenzyme, RAPD, SSR) (ISSS: Iowa Stiff Stalk Synthetic; EC: Early Canadian; W117 rel.: lines related to W 117; Iod.: Iodent; Lanc.: Lancaster; OPLac.: OP Lacaune; MYD: Mindszentpuszta Yellow Dent; Co125: Co 125 derivatives)

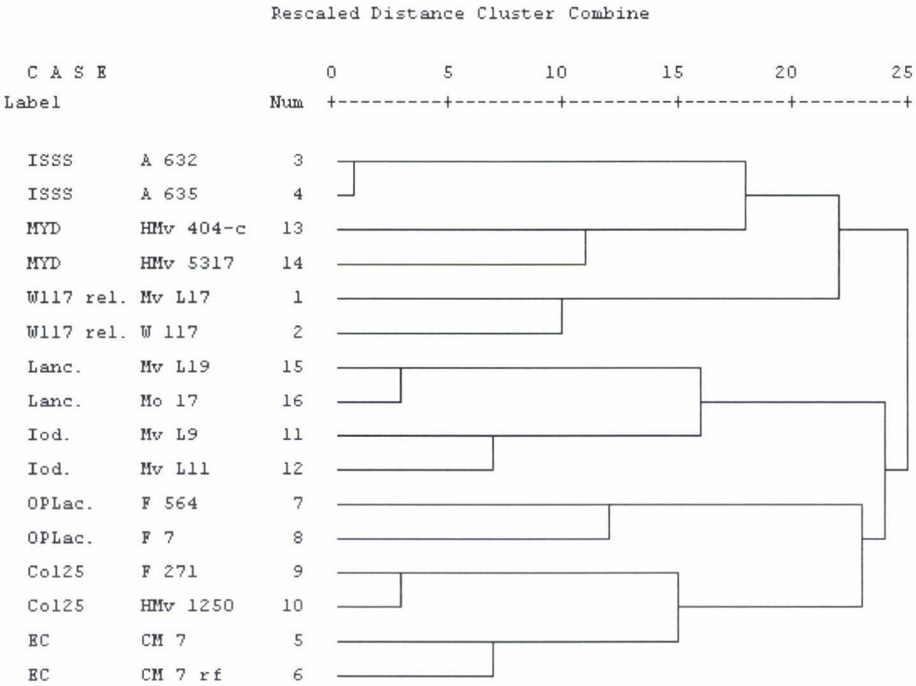

Fig. 5. Related groups on the basis of genetic markers and morphological traits (ISSS: Iowa Stiff Stalk Synthetic; EC: Early Canadian; W117 rel.: lines related to W 117; Iod.: Iodent; Lanc.: Lancaster; OPLac.: OP Lacaune; MYD: Mindszentpuszta Yellow Dent; Co125: Co 125 derivatives)

It can thus be assumed that if the genetic background of genotypes of unknown origin is to be revealed, facilitating the prediction of heterosis, analysis at the DNA level will be required, combining both dominant (RAPD, AFLP, etc.) and codominant (SSR, RFLP, isoenzyme, etc.) markers, applied together with the heterosis test, morphological descriptions and pedigree analysis.

In summary, molecular marker analyses were found to be an important part of maize breeding. They can be applied routinely to test for polymorphism, thus playing an indispensable role in checking the homogeneity of lines and hybrids and the genetic stability and purity of breeding stocks, as well as forming a basis for the protection of intellectual property rights.

The grouping of maize varieties according to their genetic background is of outstanding importance in breeding, since a sufficient genetic distance between the parental lines is a fundamental criterion for heterosis breeding. Maize lines can be put into groups which give an accurate and reliable reflection of their genetic background by combining various marker systems, supplemented with morphological data. In this way genetic markers can be of service to breeders in the planning of crossing programmes.

References

Anonymous (2001): *Protocol for distinctness, uniformity and stability tests. Zea mays* L. (Maize). European Union Community Plant Variety Office

Bourgoin-Greneche, M., Lallemand, J. (1993): Electrophoresis and its application to the description of varieties. A presentation of the techniques used by GEVES, Paris. pp. 1–63.

Dweikat, I., Ohm, H., Mackenzie, S., Patterson, F., Cambron, S., Ratcliffe, R. (1994): Association of a DNA marker with Hessian fly resistance gene H9 in wheat. *Theor. Appl. Genet.*, **89,** 964–968.

Goodman, M. M., Stuber, C. W. (1983): Races of maize. VI. Isozyme variation among races of maize in Bolivia. *Maydica*, **28,** 169–187.

Gyulai, G., Gémesné, J. A., Sági, Z., Venczel, G., Pintér, P., Kristóf, Z., Törjék, O., Heszky, L., Bottka, S., Kiss, J., Zatykó L., (2000): Doubled haploid development and PCR-analysis of F_1 hybrid derived DH-R_2 paprika (*Capsicum annuum* L.) lines. *J. Plant Physiol.*, **156,** 168–174.

Norusis, M. J. (1993): *SPSS for Windows Professional Statistics* Release 6.0, 385 pp.

Pejic, I., Ajmone-Marsan, P., Morgante, M., Kozumplick, V., Castiglioni, P., Taramino, G., Motto, M. (1998): Comparative analysis of genetic similarity among maize inbred lines detected by RFLPs, RAPDs, SSRs, and AFLPs. *Maize Genet. Coop. Newsletter*, **72,** 18–19.

Smith, J. S. C., Chin, E. C. L., Shu, H., Smith, O. S., Wall, S. J., Senior, M. L., Mitchell, S. E., Kresowitch, S., Ziegl, E. J. (1997): An evaluation of the utility of SSR loci as molecular markers in maize (Zea mays L.): comparison with data from RFLPs and pedigree. *Theor. Appl. Genet.*, **95,** 163–173.

Smith, J. S. C., Senior, M. L. (1999): The utility of simple sequence repeat (SSR) data to preferentially identify progeny lines of maize (*Zea mays* L.) that are bred from known inbred parents. *Maydica*, **45,** 205–213.

Smith, J. S. C., Smith, O. S. (1989a): The description and assessment of distance between inbred lines of maize: I. The use of morphological traits as descriptors. *Maydica*, **34,** 141–150.

Smith, J. S. C., Smith, O. S. (1989b): The description and assessment of distances between inbred lines of maize: II. The utility of morphological, biochemical, and genetic descriptors and a scheme for testing of distinctiveness between inbred lines. *Maydica*, **34,** 151–161.

Smith, J. S. C., Smith, O. S. (1992): Measurement of genetic diversity among maize hybrids: A comparison of isozymic, RFLP, pedigree and heterosis data. *Maydica*, **37,** 53–60.

Stuber, C. W., Wendel, J. F., Goodman, M. M., Smith, J. S. C. (1988): Techniques and scoring procedure for starch gel electrophoresis of enzymes from maize. *Tech. Bulletin*, **286,** 1–87.

Weining, S., Langridge, P. (1991): Identification and mapping of polymorphisms in cereals based on the polymerase chain reaction. *Theor. Appl. Genet.*, **82,** 209–216.

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GENETIC DIVERSITY TRENDS IN CENTRAL EUROPEAN HETEROTIC GROUPS

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It has been claimed that the system that delivers the products of plant breeding reduces the diversity of cultivated varieties, leading to increased genetic vulnerability. The objective of our study was to monitor the temporal trends in genetic diversity over the past five decades among maize cultivars with the largest acreage in Central Europe. Thirty individuals of five prominent open-pollinated varieties (OPVs) from Central Europe, 85 maize hybrids of economic importance, and their dent and flint parental components were examined with 55 SSRs. The genetic variation within and among varieties decreased significantly during the five decades. The five OPVs contain numerous unique alleles that were absent from the elite flint pool. Consequently, OPVs could represent useful sources for broadening the genetic base of elite maize breeding germplasm.

Key words: genetic diversity trends, European maize

Introduction

Heterotic patterns in maize have been established in germplasm adapted to temperate environments. In Central Europe hybrid breeding started in the 1950s and as a promising heterotic pattern, high-yielding US dent lines were crossed with adapted European flint lines (Schnell, 1992). During the initial phase, dent inbreds from the US were primarily selected for earliness. The steady influx of dent germplasm from North America to Europe has continued over the past 50 years. In contrast, the parental flint inbreds were developed by selfing from a few European OPVs (Reif et al., 2005a). Therefore, it can be conjectured that (1) a bottleneck occurred in the flint pool during the transition from OPVs to hybrids and (2) OPVs, which did not serve as a germplasm source for the original flint inbreds, contain untapped allelic variation useful for future breeding progress. Detailed information about a reduction in genetic diversity could help to emphasize the importance of identifying germplasm sources for broadening the established heterotic groups.

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Monitoring the genetic diversity available to farmers is important, because plant breeding practices, the registration procedures, and the marketing of new varieties could have caused a potential genetic erosion and, consequently, a potential increase in the genetic vulnerability of cultivated varieties. Snap-shots of the diversity present in maize breeding programs were reported (Messmer et al., 1992). In addition, the temporal trend in genetic diversity was investigated for a single US breeding program (Duvick et al., 2004) as well as for important public US lines (Lu and Bernardo, 2001). However, no information was available on temporal trends in the genetic diversity of the Central European heterotic pattern.

The objective of our study was to monitor the temporal trends in genetic diversity over the past five decades among maize cultivars with the largest acreage in Central Europe.

Materials and methods

Plant materials

A set of 85 maize hybrids representing the most important cultivars grown in Germany and thirty individuals of each of five prominent flint populations from Central Europe were examined (Reif et al., 2005a; b). According to the year of release the five OPVs and 85 hybrids, as well as their parents, were grouped into pre-hybrid breeding era (PH) (<1950), Period A (1951–1975), Period B (1976–1985), Period C (1986–1995) and Period D (1996–2001).

SSR analyses

DNA extraction was described in detail by Reif et al. (2005a). A total of 55 SSR markers uniformly distributed across the maize genome was used as described by Reif et al. (2005a). Details on SSR amplification, detection, and allele calling procedure were described elsewhere (Reif et al. 2005b).

Statistical analyses

In total, genotypes of 148 parental inbred lines were available. These lines were assigned to the dent and flint heterotic groups (i) according to pedigree information or (ii) by applying the K-means clustering algorithm (Hartigan and Wong, 1979), assuming two groups, if no pedigree information was available. The number of alleles (n_A), the number of new alleles (n_N), and the number of lost alleles disappearing from one period to the next (n_L) were determined.

Rogers' distances (RD) (Rogers, 1972) were determined between individual genotypes within OPVs and between individual genotypes of different OPVs. These RD estimates were averaged within each time period. In addition, the average RD between two randomly selected individuals from two different maize fields planted either with hybrid X or Y reflected the diversity between cultivated varieties. Thus, all pairwise comparisons of hybrids of a certain time period represented a measure of the diversity present at that time between cultivated varieties at an individual level and was calculated according to Reif et al. (2005a). The average expected RD between two randomly chosen individuals of one hybrid was calculated according to Reif et al. (2005a). The Wilcoxon rank sum test was applied to examine the significance between pairwise comparisons of average RDs (Hollander and Wolfe, 1973). All analyses were performed with the software Plabsoft (Maurer et al., 2004), which was implemented as an extension to the statistical software R (Ihaka and Gentleman 1996).

Results

A total of 404, 323, 328 and 337 alleles were observed in the hybrids, OPVs, dent and flint parental components, respectively. In total, 28% of the alleles present in the five OPVs were not recovered in the flint lines. Most of them (97%) had an allele frequency below 0.1 in the OPVs. The population Mahndorfer had the highest number of unique alleles (35) and Rheintaler the lowest number of unique alleles (20) not present in the flint lines.

The number of alleles (n_A) in the hybrids decreased consistently from Period A to Period D. Period B showed the highest number of both new alleles (n_N) and lost alleles (n_L). While n_A decreased for the flint parental components from Period A to Period D, with the highest loss of alleles from Period A to B, n_A increased for the dent lines from Period A to B but decreased thereafter monotonically to Period D. Across all four periods, the dent and flint lines had 65% of the alleles in common. The dent lines contained 22% and the flint lines 13% unique alleles not present in the opposite heterotic pool.

The average RD between individual genotypes was higher within OPVs (0.37) than within hybrids of Period A (0.18) (Fig. 1). The average RD between individual genotypes within hybrids decreased almost linearly from Period A to Period D. In addition, the average RD between individual genotypes of different varieties was higher for the OPVs (0.51) than for the hybrids of Period A (0.45). The average RD between individual genotypes of different hybrids increased from Period A to B but decreased from Period C to D.

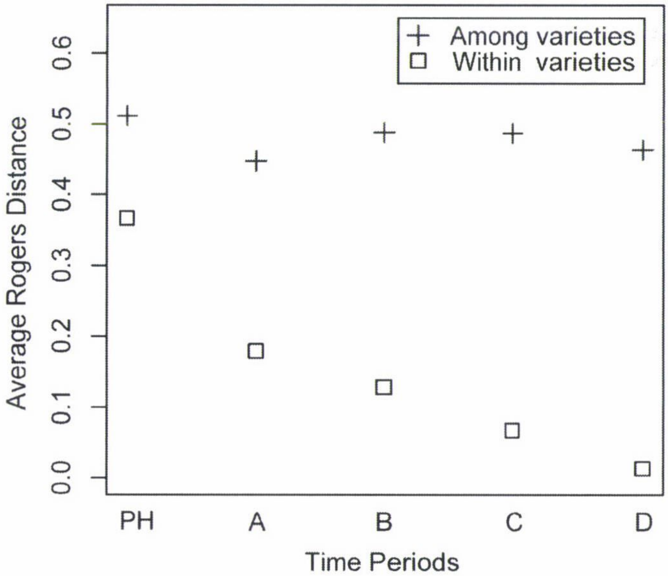

Fig. 1. Average pairwise Rogers distances based on 55 SSRs between individual genotypes within varieties and between individual genotypes among varieties grouped into pre-hybrid era PH (< 1950), period A (1951–1975), B (1976–1985), C (1986–1995), and D (1996–2001)

Discussion

Diversity during the establishment of the Central European heterotic groups

The significantly lower average Rogers distance (RD) between individual genotypes within hybrids than within OPVs reflects the reduced diversity within hybrid cultivars (Fig. 1). This can be explained by the small number of individual genotypes used to generate hybrids (Messmer et al., 1992), but also by directional selection. With increasing genetic homogeneity of varieties, a higher yield is expected but also an increase in genetic vulnerability due to reduced population buffering (Allard and Bradshaw, 1964; Hallauer et al., 1988).

Considering the total population of cultivated varieties during a certain period, the reduction in genetic diversity within varieties could have been counterbalanced by increased diversity between hybrids. However, the RD between genotypes of different varieties decreased from OPVs to hybrids. In addition, the restricted number of 5 OPVs analysed in this study presumably led to an underestimation of the diversity present in OPVs, and, thus, a considerable loss of diversity was observed within but also between varieties during the transition from OPVs to hybrids.

The large proportion of alleles from the OPVs (28) that were not recovered in the flint lines clearly indicates a reduction in allelic diversity during the establishment of the elite flint inbreds used in hybrid breeding. Apart from exotic germplasm, adapted OPVs are a promising source to broaden the genetic base of the elite flint breeding pool. The population Mahndorfer had the highest number of unique alleles (35) among the OPVs, which were absent in the flint lines. This suggests that the germplasm contribution of Mahndorfer to the elite flint pool was low. Consequently, this OPV is a very promising source for untapped allelic variation.

Diversity loss during 50 years of breeding

The genetic diversity within varieties decreased monotonically during the past 50 years (Fig. 1). Consequently, the diversity among varieties is of increased importance, but decreased slightly during the last time period. The major cause of this decrease is the multiple use of elite lines as parents for various hybrids (Reif et al., 2005a). While the multiple use of lines enables breeders to optimally exploit their elite germplasm, it inevitably leads to reduced diversity among hybrids.

The high number of unique alleles, which are exclusively present in either the dent or flint lines (35%), reflects the large divergence between the allelic profiles of the two heterotic groups (Reif et al., 2005a). The differences between the dent and flint heterotic groups already existed at the beginning of hybrid breeding in Germany. This can be explained by the long isolation between the parental germplasm sources used to establish these heterotic groups: the US dent and the European flint pool (Rebourg et al., 2003). The clear divergence of the heterotic groups at the beginning of hybrid breeding in Central Europe is in contrast to the results for US germplasm. Duvick et al. (2004) found that the US germplasm was rather unstructured in the starting phase of hybrid breeding but diverged with ongoing "reciprocal recurrent selection", as practised by testing lines in combination with testers from the opposite heterotic group.

One important criterion for the eligibility of newly bred varieties is that they are better for at least one trait (disease resistance, yield, quality) than the varieties already registered. Adding a criterion *positive contribution towards the increased genetic diversity of the available varieties*, which could be measured with molecular markers, would encourage innovative breeding. Thus, the suggested modification of the registration procedure and changes in the intellectual property practices encouraging plant breeders to introgress new diversity, could help to prevent the further potential erosion of genetic variation among commercial hybrids.

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References

Allard, R. W., Bradshaw, A. D. (1964): Implications of genotype-environment interactions in applied plant breeding. *Crop Sci.,* **4**, 503–508.

Duvick, D. N., Smith, J. S. C., Cooper, M. (2004): Long-term selection in a commercial hybrid maize breeding program. *Plant Breeding Reviews,* **24**, 109–151.

Hallauer, A. R., Russell, W. A., Lamkey, K. R. (1988): Corn breeding. In: Sprague, G. F., Dudley, J. W. (eds.), *Corn and Corn Improvement.* 3rd ed. Agron Monogr 18. ASA, CSSA and SSSA, Madison, WI. pp. 463–564.

Hartigan, J. A., Wong, M. A. (1979): A K-means clustering algorithm. *Applied Statistics,* **28**, 100–108.

Hollander, M., Wolfe, D. A. (1973): *Nonparametric Statistical Inference.* John Wiley & Sons, New York. pp. 139–146.

Ihaka, R., Gentleman, R. (1996): A language for data analysis and graphics. *J. Computational and Graphical Statistics*, **3**, 299–314.

Lu, H., Bernardo, R. (2001): Molecular marker diversity among current and historical maize inbreds. *Theor. Appl. Genet.,* **103**, 613–617.

Maurer, H. P., Melchinger, A. E., Frisch, M. (2004): Plabsoft: Software for simulation and data analysis in plant breeding. *XVIIth EUCARPIA General Congress 2004*, Tulln, Austria. pp. 359–362.

Messmer, M. M., Melchinger, A. E., Boppenmaier, J., Brunklaus-Jung, E., Herrmann, R. G. (1992): Relationships among early European maize inbreds: I. Genetic diversity among flint and dent lines revealed by RFLPs. *Crop Sci.,* **32,** 1301–1309.

Rebourg, C., Gouesnard, B., Welcker, C., Dubreuil, P., Chastanet, M., Charcosset, A. (2003): Maize introduction into Europe: The history reviewed in the light of molecular data. *Theor. Appl. Genet.,* **106**, 895–903.

Reif, J. C., Hamrit, S., Heckenberger, M., Schipprack, W., Maurer, H. P., Bohn, M., Melchinger, A. E. (2005a): Trends in genetic diversity among European maize cultivars and their parental components during the past 50 years. *Theor. Appl. Genet.,* **111,** 838–845.

Reif, J. C., Hamrit, S., Heckenberger, M., Schipprack, W., Maurer, H. P., Bohn, M., Melchinger, A. E. (2005b): Genetic structure and diversity of European flint maize populations determined with SSR analyses of individuals and bulks. *Theor. Appl. Genet.,* **111**, 906–913.

Rogers, J. S. (1972): Measures of genetic similarity and genetic distance. Studies in genetics. *VII. Univ. Tex. Publ.,* **7213**, 145–153.

Schnell, F. W. (1992): Maiszüchtung und die Züchtungsforschung in der Bundesrepublik Deutschland. *Vorträge Pflanzenzüchtung,* **22**, 27–44.

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SWISS MAIZE LANDRACES – THEIR DIVERSITY AND GENETIC RELATIONSHIPS

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Genetic variation in the flint maize (*Zea mays* L. conv. *indurata*) gene pool has decreased significantly since the introduction of hybrid breeding into Europe in the 1950s, leading to greater genetic vulnerability. Landraces, stored in gene banks, offer a valuable source to broaden the genetic basis again. The objective of this study was the genetic characterization of 166 Swiss landrace accessions originating from 7 Swiss regions (alpine valleys). The material was fingerprinted using a set of ten SSRs (Simple Sequence Repeat Markers). The resulting cladogram showed three main clusters comprising 95, 22 and 49 accessions, respectively. The largest group of accessions, from the Rhine valley of St. Gallen (RT), was present in all three main clusters. However, the majority of RT accessions was found in the first main cluster, together with those from the western neighbouring region (Linthtal) and from the southwestern neighbouring region (Wallis). Those from Tessin (southern Switzerland) were found mainly in one sub-cluster within the third main cluster. This is a very encouraging first step in appraising the genetic differences among accessions from Swiss regions.

Key words: flint maize, landraces, SSR markers, genetic distance, *Zea mays* L., Switzerland

Introduction

Maize cultivation on a worldwide scale is dominated by relatively few modern high-yielding hybrids. Although these hybrids are well adapted to a broad range of abiotic stress factors, their cultivation is still limited by cool climate and their range of adaptation may become too narrow as climatic conditions change. Furthermore, the genetic variation in the flint gene pool has decreased significantly since the introduction of hybrid breeding into Europe in the 1950s (Li, 1998; Rebourg et al., 2003; Reif et al., 2005a; Tóth and Pepó, 2003). However, tens of thousands of open-pollinated cultivars of maize are being

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maintained in gene banks (Labate et al., 2003; Warburton et al., 2002); knowledge of the extent and distribution of genetic variation within and among accessions can aid end users in choosing among them. Landraces stored in the Swiss Gene Bank at the Agroscope Changins-Wädenswil (ACW), which were collected during the phase of transition from open-pollinated varieties to hybrid breeding (1940s to 1960s), are a potential source for re-broadening the genetic basis of the flint gene pool. Molecular markers are one of the most efficient new tools for identifying genetic relationships within or between old and new varieties of a crop like maize. This approach has provided considerable insight into recent genetic developments in gene pools not only in the Corn Belt of the USA (Labate et al., 2003), but also for Europe (Reif et al., 2005b). Our objective was to characterize these accessions genetically in order to i) identify duplicates in the Gene Bank, ii) define core accessions with certain characteristics and regional adaptation and iii) find information about the distribution pathways of maize landraces in Switzerland. This information will be available to breeders to facilitate the identification of hitherto unused genetic variation in the flint gene pool.

Materials and methods

Plant material

All accessions of Swiss maize landraces available to the public in Switzerland were obtained from three sources: Most of the accessions (141) came from the Swiss Gene Bank in Changins (ACW), 21 were contributed by "Verein Rheintaler Ribelmais" (VRRM) and four came from the "Sortengarten Erschmatt" (SGE, Table 1). Included in the Swiss Gene Bank were one accession from Germany and one from France. Most of the 166 accessions were collected between 1952 and 1989, and it is assumed that they were cultivated and propagated by Swiss farmers for a long time before the introduction of modern hybrid breeding. For a comparison with modern standard maize, the hybrid Magister (Syngenta AG, Basel, Switzerland) was included as a control.

Isolation of genomic DNA

DNA was extracted from leaves of a minimum of 12 seedlings (V2 to V3 stage), frozen separately in liquid nitrogen, lyophilized for 48 h and stored at room temperature until further processing. DNA of 200 mg leaf tissue per sample was extracted with the Nucleo Spin Plant kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the standard protocol but with a few exceptions: The incubation period was prolonged up to 80 minutes and the final elution was done in two steps with 75 μL elution buffer per step to account for differences in the DNA content and in the extractability of the heterogeneous material of the landraces. The DNA concentration of the stock solutions was measured with a photometer in order to dilute the DNA concentration in the solutions to 10 ng/μL. To achieve an equal amount of DNA from individuals in the bulk analyses, aliquots of the solutions of 12 individual plants were mixed and put onto a 200 μL PCR plate.

Molecular markers

A set of 15 microsatellite markers (or simple sequence repeat markers – SSRs) was chosen based on large differences in allele size on gel images in the Maize Genome Database (MGDB, Lawrence et al., 2004). Successful amplification in most accessions was the criterion for choosing 10 markers (one per chromosome) out of this set.

Table 1

Grouping of 166 maize landrace accessions and one modern hybrid based on their origin (see also Fig. 1): Accessions RT034 to RT054 and TM 023 to TM 026 were obtained from two private Swiss initiatives, the Verein Rheintaler Ribelmais and the Sortengarten Erschmatt, respectively; all other were derived from the Swiss Federal Gene Bank (RAC, Agroscope Changins-Wädenswil)

Origin	Accessions
Germany	D
France	F
HR (Hinterrheintal)	HR01, HR02, HR03, HR04, HR05, HR06, HR07, HR08, HR09, HR10, HR11, HR12, HR13, HR14, HR15
LT (Linthtal)	LT01, LT02, LT03, LT04, LT05, LT06, LT07, LT08, LT09, LT10, LT11, LT12, LT13, LT14, LT15, LT16, LT17, LT18, LT19, LT20, LT21, LT22, LT23, LT24, LT25
PB (Puschlav-Bergell)	PB01, PB02, PB03, PB04, PB05, PB06, PB07
RT (Rheintal)	RT01, RT02, RT03, RT04, RT05, RT06, RT07, RT08, RT09, RT10, RT11, RT12, RT13, RT14, RT15, RT16, RT17, RT18, RT19, RT20, RT21, RT22, RT23, RT24, RT25, RT26, RT27, RT28, RT29, RT30, RT31, RT32, RT33, RT34, RT35, RT36, RT37, RT38, RT39, RT40, RT41, RT42, RT43, RT44, RT45, RT46, RT47, RT48, RT49, RT50, RT51, RT52, RT53, RT54
TM (Tessin)	TM01, TM02, TM03, TM04, TM05, TM06, TM07, TM08, TM09, TM10, TM11, TM12, TM13, TM14, TM15, TM16, TM17, TM18, TM19, TM20, TM21, TM22, TM23, TM24, TM25, TM26
UG (Graubünden)	UG01
VR (Vorderrheintal)	VR01, VR02, VR03, VR04, VR05, VR06, VR07, VR08
VS (Wallis)	VS01, VS02, VS03, VS04, VS05, VS06, VS07, VS08, VS09, VS10, VS11, VS12, VS13, VS14, VS15, VS16, VS17, VS18, VS19, VS20, VS21, VS22, VS23, VS24, VS25, VS26, VS27, VS28
Syngenta AG	Mag (Magister)

The PCRs were done with thermocyclers (Mastercycler ep gradient or Mastercycler, Eppendorf, Hamburg, Germany) using Taq DNA polymerase with advanced buffer and desoxynucleotide sets (Eppendorf, Hamburg, Germany). A protocol of the MGDB (MGDB: Maize mapping project – SSR protocols) was adjusted to the landrace material and the primers used: 20 μL of reaction mixture contained one unit of Taq polymerase, 50 ng DNA, 1.6 μL dNTP mixture (final concentration of each dNTP 80 mM), 0.25 μM of primer mix, 1× Taq advanced buffer and sterile, deionised water (dd H_2O).

The annealing temperatures were tested for each primer separately before running the analyses. The thermocycler program consisted of an initial denaturation step at 95°C for 2 min followed by 34 cycles (35 cycles if using the Mastercycler) of 94°C for 15 to 60 s, 15 to 60 s at annealing temperature and 30 to 60 s at 72°C. The final step was an additional extension period of 5 min at 72°C.

Gel electrophoresis

To separate, document and compare the products obtained by PCR reactions, agarose gel electrophoresis (Senior and Heun, 1993; Sharopova et al., 2002) was used. The amplification products and a 20 bp molecular ruler (Bio-Rad Laboratories, Hercules CA, USA) as size standard were run for 105 min at 150 V on a 4% (w/v) agarose gel (Agarose 50 – 1000 Bp, Carl Roth, Karlsruhe, Germany). PCR products from DNA bulks of the different accessions were each loaded into one lane. The different accessions were adjacent on each gel (192 samples per gel) to enable the identification of different alleles, even in closely related accessions. Then, the gels were stained in an EtBr-solution with 1 mg/L EtBr for 15 min. After rinsing them for 15 min in dd H_2O, the gels were photographed (Doc Print, Vilber Lourmat, Torcy Z. l. Sud, France). The resulting image files were processed with Gel-Pro Analyzer (Media Cybernetics Inc., Silver Spring MD, USA) to determine the fragment size of the PCR products.

Statistics

By using Gel-Pro Analyzer it was possible to measure the relative intensities of the bands in one lane and thus estimate the frequency of a given allele in a population as described elsewhere (Reif et al., 2005b). The Shared Allele Distance (Chakraborty and Jin, 1993) and the Rogers distance (Rogers, 1972; 1991) were calculated based on the estimated allele frequencies using powermarker (Liu, 2001). The Rogers Distance assumes no knowledge about the evolutionary forces at work and possesses properties that make it suitable for the identification of duplicates in seed banks (Reif et al., 2005c). The shared allele distance is also not based on assumed stepwise mutation and is suitable for individuals and populations. The Mantel test (Mantel, 1967) was done to determine the correlation between the two genetic distance matrices.

The shared allele distance matrix served as input for constructing a rectangular cladogram using the Neighbor Joining (NJ, Saitou and Nei, 1987) method. NJ is effective for large data sets. It is not based on the assumption of a similar rate of evolution of all lineages and produces an unrooted tree.

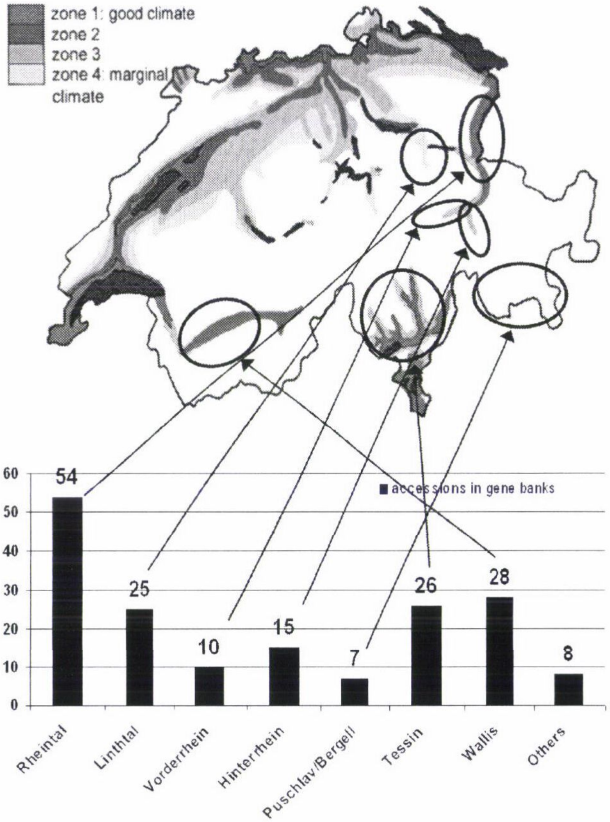

Fig. 1. Map of Switzerland. Differences in climate are represented by different shades of grey (see legend). Climatic conditions indicate suitability for maize cultivation. Regions of origin of maize accessions are represented by ovals. For further information see Table 1

Results

Almost all the collected landraces were developed in geographically isolated mountain valleys with a fairly favourable climate for maize cultivation (Fig. 1). The regions can be roughly separated into the northern alpine valleys with a submontane to montane central European climate (Rhine valleys and Linthtal), the central alpine valleys with a dry, submontane climate (Wallis) and the southern alpine valleys with a submontane-insubric climate, characterized by high precipitation and high temperature (Tessin, Puschlav and Bergell). The northern, central and southern alpine valleys are separated by high mountain ridges. The most productive northern region is probably the Rhine valley of St. Gallen, where the largest number of landraces was collected and, probably, cultivated. The adjacent region to the west, Linthtal, also contributed a considerable number of accessions, as well as the upstream valleys, Vorderrhein and Hinterrhein. A large collection from the central Alps originated from the Wallis, which has an exceptionally dry climate. South of the Alps, the largest collections came from Tessin.

In all, 131 alleles were identified, using the bulk of the landrace accessions and the modern hybrid. This corresponds to a mean of 13.1 alleles per locus ranging from 9 to 20, including one allele for non-amplification (null), in case it occurred in the respective accession. The Mantel test showed a very high correlation of 0.969 between Rogers Distance and the Shared Allele Distance ($p<0.001$). Therefore, only the cladogram, based on Shared Allele Distance (Fig. 2), is shown.

According to this cladogram, the largest group of accessions from the Rhine Valley of St. Gallen (RT) was genetically the most heterogeneous, distributed over all main and sub-clusters down to the third hierarchical level; however, the majority of the accessions was found in the first main cluster. Here almost all the accessions of the adjacent region to the west, Linthtal (LT), were also grouped very closely together, with just a few mavericks in the other two main clusters. The fewer accessions from the Hinterrhein valley (HR) were usually grouped in the first main cluster but were not as close to each other as the LT accessions. However, some belonged to the few that were grouped together with some of the RT landraces and most of the landraces from the Vorderrhein valley (VR). Three accessions from VR were in the lower part of the third cluster, similar to a large number of Tessin (TM) accessions and a few of the RT accessions.

The accessions from south of the Alps were at very different places on the cladogram. Despite a few escapees, those from Wallis were grouped very closely together in the first main cluster, similar to the LT accessions, although distinctly separated from them. By contrast, those from Tessin were usually found in one region of the third main cluster with just a few escapees in the first cluster. The smallest group from the third southern region, Puschlav/Bergell (PB), was also distributed at these two extreme positions in the first and last main clusters.

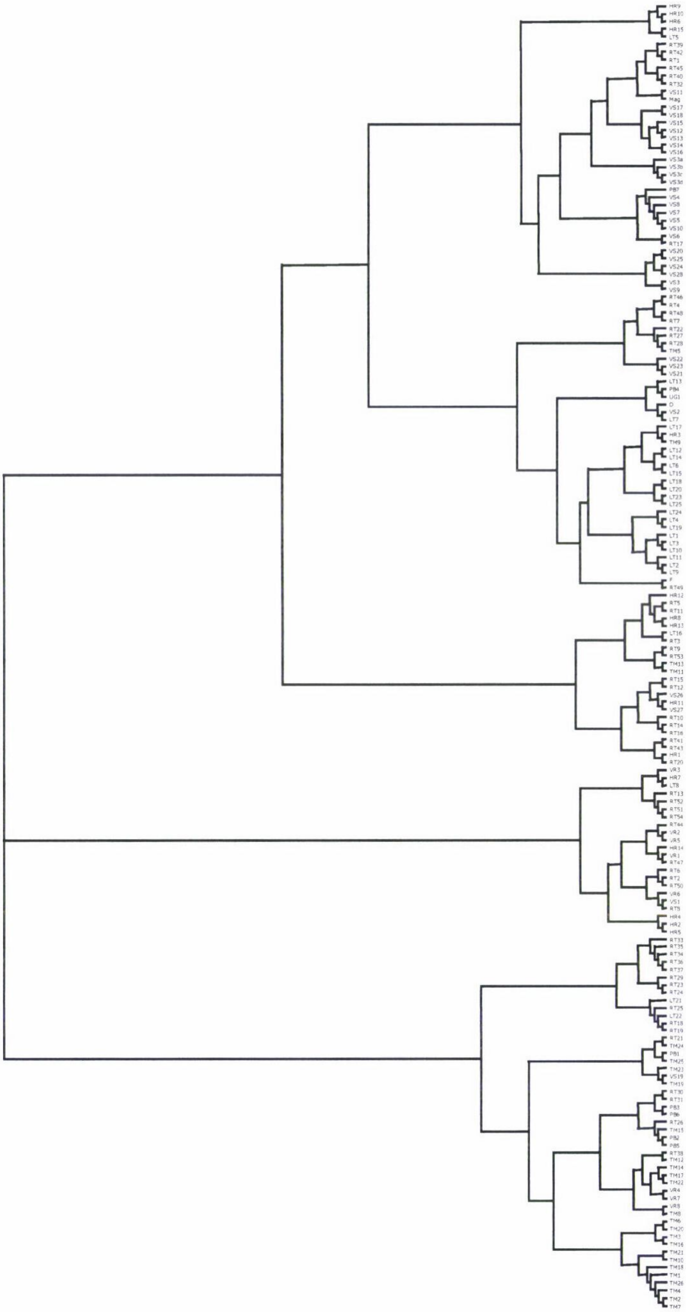

Fig. 2. Rectangular cladogram showing shared allele distance proportions, calculated on the basis of estimated allele frequencies. Each accession was represented by a DNA bulk of no more than twelve plants. For information on accession names, see Table 1

Discussion

It has been shown that bulk analysis, using a well-defined set of markers, clearly distinguishes among the large set of 166 Swiss maize landraces from the national gene bank. This is a very encouraging first step in appraising the genetic differences in accessions from regions north and south of the Alps. As described above, there are clear indications that accessions collected from regions like Wallis, central Alps, and the Linthtal, north of the Alps, are each genetically much closer together than collections from a very productive region with a well-documented, long-standing tradition of maize cultivation, such as the Rhine valley of St. Gallen.

Information derived from the analysis of genetic proximity may reveal overlap between groups of different origin. First indicators were found that small groups in the central Alps may contain material both from the north and the south, whereas regions in the southern Alps and central Alps have a dissimilar genetic background, as clearly proved for the accessions from Tessin and Wallis, respectively. The grouping within the first main cluster of the cladogram and agronomic early vigour tests (Peter et al., 2006) indicate the possibility of a close relationship between one clearly defined set of RT accessions and the three control varieties, the standard hybrid Magister and the German landrace (D). This is a first indicator that the Swiss contribution to the European Flint Maize Pool is from a narrow section of the total Swiss Gene Pool, as represented by the landraces. Thus it is well worthwhile preserving and further analysing the remaining rich heritage of landraces.

It remains to be determined phenotypically or genetically with individual plants or more markers whether the proximity in the accessions mentioned above is a reliable indicator for identification that would warrant the exclusion of doublets. The agronomic traits of all entries will be tested in simple field trials; it is anticipated that agronomic groups matching genetic groups will be found.

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References

Chakraborty, R., Jin, L. (1993): Determination of relatedness between individuals using DNA-fingerprinting. *Human Biology*, **65,** 875–895.

Labate, J. A., Lamkey, K. R., Mitchell, S. E., Kresovich, S., Sullivan, H., Smith, J. S. C. (2003): Molecular and historical aspects of corn belt dent diversity. *Crop Sci.*, **43,** 80–91.

Lawrence, C. J., Dong, Q., Polacco, M. L., Seigfried, T. E., Brendel, V. (2004): MaizeGDB, the community database for maize genetics and genomics. *Nucl. Acids Res.*, **32,** D393–397.

Li, Y. (1998): Development and germplasm base of maize hybrids in China. *Maydica*, **43,** 259–269.

Liu, J. (2001): PowerMarker: new genetic data analysis software. Version 3.0. Free program distributed by the author over the internet from: http://www.powermarker.net.

Mantel, N. (1967): Detection of disease clustering and a generalized regression approach. *Cancer Research*, **27,** 209

Peter, R., Eschholz, T. W., Stamp, P., Liedgens, M. (2006): Swiss maize landraces – early vigour adaptation to cool conditions. *Proc. 20th Conf. Maize and Sorghum Sect. EUCARPIA*, *Acta Agron. Hung.,* **54**, 329–336.

Rebourg, C., Chastanet, M., Gouesnard, B., Welcker, C., Dubreuil, P., Charcosset, A. (2003): Maize introduction into Europe: the history reviewed in the light of molecular data. *Theor. Appl. Genet.*, **106,** 895–903.

Reif, J., Hamrit, S., Heckenberger, M., Schipprack, W., Maurer, H., Bohn, M., Melchinger, A. (2005a): Trends in genetic diversity among European maize cultivars and their parental components during the past 50 years. *Theor. Appl. Genet.*, **111,** 838–845.

Reif, J. C., Hamrit, S., Heckenberger, M., Schipprack, W., Maurer, H. P, Bohn, M., Melchinger, A. E. (2005b): Genetic structure and diversity of European flint maize populations determined with SSR analyses of individuals and bulks. *Theor. Appl. Genet.*, 111, 906–913.

Reif, J. C., Melchinger, A. E., Frisch, M. (2005c): Genetical and mathematical properties of similarity and dissimilarity coefficients applied in plant breeding and seed bank management. *Crop Sci.*, **45,** 1–7.

Rogers, J. S. (1972): Measures of genetic similarity and genetic distance. *Studies in Genetics VII*, University of Texas Publication 7213, Austin, TX. pp. 145-153.

Rogers, J. S. (1991): A comparison of the suitability of the Rogers, Modified Rogers, Manhattan, and Cavalli-Sforza and Edwards distances for inferring phylogenetic trees from allele frequencies. *Systematic Zoology*, **40,** 63–73.

Saitou, N., Nei, M. (1987): The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, **4,** 406–425.

Senior, M. L., Heun, M. (1993): Mapping maize microsatellites and polymerase chain reaction confirmation of the targeted repeats using a CT primer. *Genome*, **36,** 884–889.

Sharopova, N., et al. (2002): Development and mapping of SSR markers for maize. *Plant Molecular Biology*, **48,** 463–481.

Tóth, S., Pepó, P. (2003): Studies on basic maize breeding stocks. *Növénytermelés*, **52,** 609–621.

Warburton, M. L., Xianchun, X., Crossa, J., Franco, J., Melchinger, A. E., Frisch, M., Bohn, M., Hoisington, D. (2002): Genetic characterization of CIMMYT inbred maize lines and open pollinated populations using large scale fingerprinting methods. *Crop Sci.*, **42,** 1832–1840.

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SWISS MAIZE LANDRACES – EARLY VIGOUR ADAPTATION TO COOL CONDITIONS

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Due to their good early vigour, Swiss maize landraces have been used extensively to develop the Flint Pool of European hybrid-breeding programmes. However, the basis of good early vigour, especially under cool conditions, has not been elucidated. Of 166 pre-screened Swiss maize landraces, 17 contrasting accessions were tested together with two control accessions, a German landrace and a modern hybrid cultivar with proven good early vigour, at sites in the midlands and the foothills of the Alps in Switzerland. To investigate early vigour, photosynthesis, leaf greenness and plant growth were recorded. Compared to the modern standard hybrid cultivar, northern accessions showed superior early vigour under cold stress in the field for all traits examined in these experiments, whereas these traits were much less pronounced in southern accessions. In particular, some accessions from the Rhine valley seem to be promising sources of early vigour for use in breeding programmes. These findings support the hypothesis that long-term selection resulted in the adaptation of maize landraces to their local environment. Compared to the phylogenetic tree, it is evident that accessions with superior early vigour are related to each other and originated in the Rhine valley.

Key words: flint maize, landraces, early vigour, cold tolerance, *Zea mays* L.

Introduction

The oldest drawing of a maize plant in Europe can be found in the well-known book by the botanist Leonhart Fuchs (Fuchs, 1549). This is accepted as proof that this neophyte was known in parts of Switzerland, Germany and France at that time. The drawing clearly depicts a tillered Northern Flint type, although the early introductions of this type are believed to have been tall untillered races from the Caribbean. According to Rebourg et al. (2003), there were two main introductions of these maize types into Europe, but their origin is still unknown and will probably remain so.

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According to definition, landraces have been selected and multiplied by farmers over centuries; as a result, they are well adapted to their natural and anthropological environments and can tolerate several biotic and abiotic stresses (Zeven, 1998). This was a prerequisite for landraces to spread within Europe, making it a valuable and common crop in some areas of the continent (compare Brandolini, 1971). In northeastern Switzerland their cultivation probably began as early as 1600 in the upper Rhine valley, favoured by frequent warm and dry southern winds ("Föhn"). Thus, in St. Gallen, maize was already the most important cereal in the 18th century and the population had developed specific dishes such as a particular maize porridge (Ribel), which preferably needs cultivars with white grains for preparation. This typical Northern Flint (*Zea mays* L. conv. *indurata*) material was adapted by farmers to a range of valleys along the Rhine, which demand specific adaptations because spells of cold temperatures from the north are frequent until the early summer and because the "Föhn" can give rise to prolonged periods of drought. It is not known whether the late-maturing germplasm from Tessin in the southern part of the Alps belongs to the same genetic group or whether it was introduced from northern Italy (compare Lucchin et al., 2003). After the Second World War, the intensification of agriculture in Switzerland led to a rapid loss of interest in the cultivation of landraces which had hitherto been cultivated and maintained by farmers (Barcaccia et al., 2003). Fortunately, far-sighted maize breeders took the initiative in the early 1950s to start a comprehensive collection, now stored at the gene bank of the Federal Research Station Agroscope Changins-Wädenswil.

Although some of the landraces, mostly from the northern part of the Swiss Rhine valley, are documented as being the forerunners of the modern Swiss or European Flint Pool, it is difficult to assess whether a full agronomic evaluation was achieved in the early days of hybrid breeding. This particularly concerns the early growth, until the end of the heterotrophic period, which ends between the 3-leaf and the 5-leaf stage (Gay et al., 1990). Within the scope of a Swiss National Action Plan (NAP) for the rescue and reuse of genetic resources, a concerted effort is being made to gain a better understanding of the intrinsic value of gene bank resources. For the maize landraces, a programme was launched with the following hypotheses:

– Swiss maize landraces are adapted to the specific conditions of their site of origin.

– Landraces adapted to marginal (cool) conditions during early development can be found within this material.

Materials and methods

Eighteen pre-selected Swiss maize landrace accessions were tested together with a leading standard hybrid cultivar Magister (Table 1) in six environments for two years. In 2004, all the accessions were planted at two sites in the Swiss midlands (Wiesendangen, 450 masl; Lindau 550 masl) and at two sites in the foothills of the Alps (Magdenau, 830 masl; Degersheim 870 masl) to subject them to different levels of cold stress in spring. Single-row test plots (7 m long and 0.75 m between rows) were isolated by a small, stable hybrid cultivar (Monopol). In 2005, isolated two-row test plots, 6 m long, were sown at one site in the midlands (Lindau, 550 masl) and another site in the foothills of the Alps (Magdenau, 870 masl). Sowing was done as early as possible (2004: April 22 at Lindau and Wiesendangen and April 28 at Magdenau and Degersheim; 2005: May 1 at Lindau and May 2 at Magdenau) to expose seedlings to cold stress during the first weeks of development. The seeds were dressed with imidacloprid (Gaucho, Bayer CropScience, Monheim, Germany) and sown 0.15 m apart, at a soil depth of 0.06 m and a density of 8.9 plants m^{-2}. Plant protection and fertilization were applied according to good agricultural practice.

Measurements

To analyse early development a series of parameters were measured. During a cold spell just before the plants reached the 3-leaf stage in 2004 and the 4-leaf stage in 2005, the reaction of the photosynthetic apparatus was evaluated by measuring leaf greenness and photosynthesis. Leaf greenness measurements (Minolta SPAD-502 chlorophyll meter, Minolta Corporation, Ramsey, MN, USA) were recorded twice on the third leaf of ten plants per plot. The maximum quantum efficiency of photo system II (PSII) primary photochemistry (F_v/F_m) was determined for the last fully developed leaf of 10 dark-adapted plants per plot by means of a pulse amplitude modulation fluorometer (PAM-2000, Walz, Effeltrich, Germany). Destructive shoot samplings were done on 0.975 m^2 (1.3 m row length) at the 3-leaf and 6-leaf stage of the standard hybrid cultivar Magister (Syngenta Seeds AG, Basel, Switzerland). Fully developed leaves per plant were counted and the samples were divided into leaf blades and stalks. Leaf area was determined subsequently with a leaf area meter (LI-COR 3100, Lincoln, NE, USA) and the shoot fractions were dried at 105°C for 48 h to constant mass. To assess the general early vigour of the shoots, the plots were visually scored on a scale of 1 (highly vigorous) to 9 (very weak) with regard to shoot mass and leaf colour. The average of four scorings in the first 30 days after planting were taken into account in subsequent analyses. After the standard hybrid cultivar exhibited a black layer on the grains, plants from a 3 m row were harvested and the total dry matter yield and kernel dry matter yield were determined.

Table 1

Subset of Swiss landrace accessions used in this study[1]

Code	Accession No.
Magister	Syngenta seeds AG Basel
D	ZM127
LT007	ZM059
LT016	ZM055
LT021	ZM089
HR006	ZM007
VR006	ZM134
RT001	ZM018
RT032	ZM103
RT039	VRRM 6
RT040	VRRM 7
RT045	VRRM 12
RT048	VRRM 15
PB007	ZM125
TM011	ZM073
TM013	ZM075
TM023	EM001
TM026	EM004
VS001	ZM023

[1] For complete information see: Eschholz et al. (2006); Code: accession code used in this study; Accession No.: No. in the Swiss National Genebank

Statistical analysis

Plots from all the experimental sites were arranged in a randomized complete block design with four replications. The statistical model used for the analysis of variance (ANOVA) was a linear mixed model of the form:

$$Y_{ijk} = \mu + env_i + acc_j + bl(env)_{k(i)} + env^*acc_{ij} + E_{ijk}$$

where env_i and acc_j are the fixed effects of the ith environment and the jth accession, respectively; $bl(env)_{k(i)}$ represents the kth random block effect nested within the ith environment and E_{ijk} is the residual error term. Data were transformed as required to meet the assumptions of normality and homogeneity of variance with regard to the residuals. The analyses were run using SAS® PROC MIXED (Littell et al., 2006). Pearson correlation coefficients were calculated in the computing language R (R Development Core Team, 2005) on untransformed data using the rcorr function from the package Hclust (Harrell, 2006).

Unless otherwise stated, a level of significance of 0.05 was assumed.

Results

As shown in Table 2, the statistical analysis indicates a clear impact of the environment (ENV) and the accessions (ACC) on seedling and juvenile traits up to the 6-leaf stage; interactions existed for the leaf traits but not for biomass accumulation at these stages. The total leaf area was very closely related to shoot dry matter at the 3-leaf stage and will not be discussed further.

Good early vigour ratings were observed for a group of Rhine valley (RT) accessions. These were slightly superior to the vigour of the standard variety (Table 3). Contrastingly, most of the southern accessions, from Puschlav (PB), Wallis (VS) and especially from Tessin (TM), were rather weak in this regard. This comprehensive rating indicated that considerable variability exists for early vigour in this subset of Swiss accessions.

At the 3-leaf stage, the photosynthetic efficiency of PSII (F_v/F_m) varied slightly between accessions. However, due to the recent cold spell, the values were considerably below the typical value for unstressed plants (0.8). The narrow range for F_v/F_m values may be a consequence of similar reaction amplitudes for this parameter in the photosynthetic apparatus of this material. The lowest values tended to be for the accessions from Tessin. The same was true for leaf greenness (SPAD); in contrast, most of the other accessions showed fairly high values for these traits.

Table 2

Significances according to F-test of 5 main parameters for the two main factors environment (ENV) and accession (ACC) and their interaction

	VIG	F_v/F_m	SPAD	DM3	DM6
ENV	*	***	***	***	***
ACC	***	***	***	***	***
ENV×ACC	*	***	***	n.s.	n.s.

*, ** and ***: significant at the 5%, 1% and <1% level of probability, respectively; VIG: early vigour index, F_v/F_m: efficiency of photosynthesis, SPAD: leaf greenness (SPAD-index), DM3: dry matter at the 3-leaf stage (g m^{-2}), DM6: dry matter at the 6-leaf stage (g m^{-2}); n.s. = non-significant

Table 3

Means over 6 environments and % of the standard hybrid cultivar (MAGI) for 5 parameters describing the early vigour of 18 landrace accessions and 1 modern hybrid cultivar (MAGI)

Accession	VIG		F_v/F_m		SPAD		DM3		DM6	
	Index	%	Index	%	Index	%	Index	%	Index	%
MAGI	2.53	100	0.55	100	26.3	100	2.48	100	27.0	100
D	2.40	95	0.56	103	27.4	104	2.44	98	27.3	101
LT007	2.88	113	0.55	101	26.8	102	2.14	86	27.5	102
LT016	2.60	103	0.56	103	27.6	105	2.42	97	33.3	123
LT021	3.31	131	0.54	98	25.5	97	2.27	92	21.1	78
HR006	3.02	119	0.54	98	25.8	98	1.83	74	24.2	90
VR006	2.37	94	0.55	101	27.9	106	2.33	94	32.6	121
RT001	2.51	99	0.53	97	27.0	103	2,53	102	29,3	109
RT032	2.11	83	0.55	101	26.5	101	2.31	93	28.0	104
RT039	1.68	66	0.55	101	27.4	104	2.50	101	33.4	124
RT040	1.96	77	0.53	96	25.1	96	2.54	102	29.4	109
RT045	2.17	86	0.57	103	27.9	106	2.83	114	31.8	118
RT048	2.19	87	0.58	106	27.9	106	2.55	103	34.2	127
PB007	3.33	131	0.54	98	26.5	101	1.53	62	20.6	76
TM011	3.98	157	0.53	96	25.6	98	1.77	71	22.2	82
TM013	4.05	160	0.52	95	25.0	95	1.91	77	20.2	75
TM023	4.38	173	0.50	91	25.4	97	1.57	63	21.7	81
TM026	3.06	121	0.53	96	24.4	93	2.25	90	25.7	95
VS001	3.27	129	0.57	104	28.7	109	1.80	72	23.0	85
Minimum	1.68		0.50		24.4		1.53		20.2	
Maximum	4.38		0.58		28.7		2.83		34.2	

VIG: early vigour index, F_v/F_m: efficiency of photosynthesis, DM3: dry matter at the 3-leaf stage (g m^{-2}), SPAD: leaf greenness (SPAD-index), DM6: dry matter at the 6-leaf stage (g m^{-2}). Accession: see Table 1 and compare Eschholz et al. (2006)

A number of similarities were detected between the early vigour rating and the early accumulation of shoot biomass at the 3-leaf stage, but the error variance was higher for the latter trait. Again the values were poorer for accessions from south of the Alps (PB, TM and VS accessions). All the Rhine valley accessions stood out once more with good shoot development, but the values were not significantly higher than those for the standard hybrid cultivar and the other northern accessions.

By the 6-leaf stage, differences in shoot dry matter had become much stronger between the accessions; LT16, VR06, RT39, RT45 and RT48 had achieved about 20% higher values than Magister, whereas all the accessions from south of the Alps had up to 25% lower values than the control. A remarkable increase in biomass (up to 14-fold) compared to that at the 3-leaf stage was recorded within the 3 weeks between the first and the second measurements. Two northern accessions, LT21 and HR06, started with weak values for general vigour and performed throughout this early developmental phase in a similar manner to the southern accessions. The southern accession VS was outstanding and had good values for leaf greenness and photosynthetic efficiency, as did the northern accessions, but slow early growth, like the accessions from Tessin.

The relationship between the studied traits (Table 4) varied somewhat between environments. Considering all six environments, the correlations were rarely very close, with the self-explanatory exception between the shoot biomass at the 3-leaf and 6-leaf stages, which were both well correlated with the photosynthetic efficiency at the 3-leaf stage. Some weak but significant correlations with total shoot biomass at maturity (DMM) were found, but must be regarded with caution due to the different origin of the germplasm.

Table 4

Pearson correlation coefficients (r) with data of 6 environments

	VIG	F_v/F_m	DM3	SPAD	DM6
VIG	–	–	–	–	–
F_v/F_m	–0.14*	–	–	–	–
DM3	–0.35***	0.43***	–	–	–
SPAD	–0.06	0.17**	–0.25***	–	–
DM6	–0.23***	0.6***	0.72***	–0.03	–
DMM	–0.29***	0.27***	0.43***	–0.27***	0.36***

*, ** and ***: significant at the 5%, 1% and <1% level of probability, respectively; VIG: early vigour index, F_v/F_m: efficiency of photosynthesis, DM3: shoot dry matter at the 3-leaf stage (g m^{-2}), SPAD: leaf greenness (SPAD-index), DM6: shoot dry matter at the 6-leaf stage (g m^{-2}), DMM: shoot dry matter at maturity (g m^{-2})

Discussion

Genetic germplasm, temporal exotism

During the last 130 years, the early vigour of maize varieties seems to have increased, as indicated by the comparison of the growth of a selection of old (1875) and newer (1976) German varieties (Stamp and Kullman, 1984). This can be explained by the important inclusion of germplasm from the Rhine valley of St. Gallen in modern breeding material, because this material is, on average, superior to the modern standard cultivar as well as to the southern German accession (D) from the upper Rhine valley of Freiburg. Thus, using germplasm from different periods of time, referred to as temporal exotism, seems to be of value mainly when the clear focus is on a trait like early vigour, as in the present study. Previous studies showed that, in the field, the superior early vigour of spatially exotic germplasm from high altitudes in Mexico (Stehli et al., 1999) was due in part to a smaller decrease in the maximum quantum efficiency of photosystem II primary photochemistry (F_v/F_m) (Leipner et al., 1999). However, despite the proven importance of this photosynthetic parameter in modern selection programmes for improved cold tolerance (Fracheboud et al., 1999), the differences in this trait were insufficient both between accessions and between accessions and the modern standard cultivar in the environments investigated here; thus, special attention to such traits is not merited on the basis of the selected landraces. Nevertheless, this study shows the usefulness of combining traits that can be measured rapidly and inexpensively to describe larger sets of accessions with regard to their early vigour.

Genetic relationships and early vigour

The accessions tested here for early vigour were selected without any knowledge of the genetic relationship. For this reason, the choice was based solely on physiological and agronomic data. A preliminary comparison with the newly developed phylogenetic tree (compare Eschholz et al., 2006) reveals that some of the accessions tested in the reported field studies do not represent the core of their region of origin. The most important group of accessions from the Rhine valley of St. Gallen (RT) was chosen for their good early vigour in preliminary agronomic tests. Although these accessions were represented all over the map, the six accessions investigated were all assembled in a single genetic cluster. From the western Linthtal valley, two representatives with acceptable early vigour belong to the same cluster, whereas the third accession, LT021, clusters genetically with southern accessions from Tessin and shows rather low vigour. Care should be taken, however, when speculating about such relationships between genetic origin and performance; by chance, the single representatives for HR and PB and two of the four representatives from Tessin (TM011 and TM013) belong to the same genetic cluster as the six RT accessions without there being a positive effect on field performance. Only two accessions, VR006 and VS001, belong to a second main genetic cluster; both show moderate to low early vigour but VS001 is quite robust with respect to leaf photosynthetic parameters.

The two control accessions, the modern standard hybrid cultivar and the old German landrace (D), were very similar in all parameters related to vigour, usually below the RT accessions; both were in the same genetic cluster as these landraces.

First conclusions

– There are significant differences in important parameters of landrace accessions with regard to early vigour under cool conditions.

– Material from northern and southern Switzerland can be distinguished clearly by these parameters. It is assumed that these two pools have different genetic backgrounds.

– Some accessions from the Rhine valley show superior early development compared to a standard modern hybrid cultivar.

– Considerable diversity has been found within and between accessions; this merits a closer analysis of specific new traits for early vigour.

– Good early development may have an impact on biomass production at harvest.

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References

Barcaccia, G., Lucchin, M., Parrini, P. (2003): Characterization of a flint maize (*Zea mays* L. var. *indurata*) Italian landrace. II. Genetic diversity and relatedness assessed by SSR and Inter-SSR molecular markers. *Genet. Resour. Crop Evol.*, **50,** 253–271.

Brandolini, A. (1971): Preliminary report on South European and Mediterranean maize germplasm. In: Kovács, I. (ed.), *Proc. 5th Conf. Maize and Sorghum Sect. EUCARPIA*. Akadémiai Kiadó, Budapest, Hungary, p. 290.

Eschholz, T. W., Peter, R., Stamp, P., Hund, A. (2006): Swiss maize landraces – their diversity and genetic relationships. *Proc. 20th Conf. Maize and Sorghum Sect. EUCARPIA*, *Acta Agron. Hung.*, **54**, 321–328.

Fracheboud, Y., Haldimann, P., Leipner, J., Stamp, P. (1999): Chlorophyll fluorescence as a selection tool for cold tolerance of photosynthesis in maize (*Zea mays* L.). *J. Exp. Bot.*, **50,** 1533–1540.

Fuchs, L. (1549): *De Historia Stirpium Commenarii Insignes*. Lugduni: apud Barthazarem Arnolletum, Lyon, France, p. 841.

Gay, J. P., Goytino, B., Tollenaar, M. (1990): Évolution comparée du système aérien et racinaire au stade jeune. In: Picard, D. (ed.), *Communications au Colloque: La vie du maïs, physiologie du maïs, application à la production*, INRA, Paris, pp. 91–99.

Harrell, F. E. (2006): Hmisc: Harrell Miscellaneous. R package version 3.0–12.

Leipner, J., Stehli, A., Soldati, A. (1999): Photosynthetic performance of exotic maize (*Zea mays* L.) germplasm from tropical highlands at low and high temperature. *J. Appl. Bot.*, **73,** 20–24.

Littell, R. C., Milliken, G. A., Stroup, W. W., Wolfinger, R. D., Schabenberger, O. (2006): *SAS® System for Mixed Models*. SAS Inst., Cary, 814 p.

Lucchin, M., Barcaccia, G., Parrini, P. (2003): Characterization of a flint maize (*Zea mays* L. convar. *mays*) Italian landrace: I. Morpho-phenological and agronomic traits. *Genet. Resour. Crop Evol.*, **50,** 315–327.

R Development Core Team (2005): *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria.

Rebourg, C., Chastanet, M., Gouesnard, B., Welcker, C., Dubreuil, P., Charcosset, A. (2003): Maize introduction into Europe: the history reviewed in the light of molecular data. *Theor. Appl. Genet.*, **106,** 895–903.

Stamp, P., Kullman, A. (1984): Shoot growth, green leaf area development and NAR of maize. Comparison between cultivars grown in Germany 1875 and 1976. *Maydica*, **29,** 235–246.

Stehli, A., Soldati, A., Stamp, P. (1999): Vegetative performance of tropical highland maize (*Zea mays* L.) in the field. *J. Agron. Crop Sci.*, **183,** 193–198.

Zeven, A. C. (1998): Landraces: A review of definitions and classifications. *Euphytica*, **104,** 127–139.

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COMBINING ABILITIES AND GENETIC RESEMBLANCE OF MAIZE INBRED LINES

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A diallel cross between ten maize inbred lines was carried out to estimate genetic parameters for grain yield and determine the heterosis and combining abilities of the inbreds and their crosses. Highly significant values for both GCA and SCA were found concerning this trait.

The genetic distance (GD) of ten inbreds was evaluated using protein and RAPD markers. The GD based on protein markers ranged from 0.094, found between two lines of the same origin, up to 0.359 between two pairs of inbreds originating from different heterotic groups. Similar results were obtained with RAPD, where both extremes were found among the same F_1 combinations.

The reliability of the application of molecular markers was confirmed by the highly significant values of the correlations between GD/heterosis and GD/SCA, especially based on RAPD.

Key words: combining abilities, heterosis, molecular markers, RAPD

Introduction

Information on the heterotic patterns and combining ability among maize germplasm is essential in maximizing the effectiveness of hybrid development (Beck et al., 1990). The choice of the most efficient breeding programme depends on the knowledge of the type of gene action involved in the inheritance of important traits such as grain yield and its components.

Besides field testing methods and pedigree data, the use of molecular markers provides breeders with essential information on the genetic identification of inbred lines and maize populations. Molecular markers allow a direct comparison of the similarity of genotypes at the molecular level (Drinić-Mladenović et al., 2004). Several analyses of maize genetic variability have been performed using molecular markers to obtain genotype characterization (Gethi et al., 2002), to assign lines to heterotic groups (Melchinger et al., 1992), or to estimate the heterosis among inbred lines (Drinić-Mladenović et al., 2002). The RAPD technique has been useful in studying polymorphism, identifying genes of interest, and characterizing maize inbred lines (Hahn et al., 1995; Heun and Helentjaris, 1993) and hybrids (Stojšin et al., 1996; Sun et al., 2001). On the other hand, the polymorphism of protein markers has proved to be a useful preliminary method for the characterization of maize inbred lines (Wang et al., 1994).

Materials and methods

The study included five medium early and five medium late maize inbred lines, originating from the BSSS (3 inbreds), Lancaster (4 inbreds), Wf 9 (1 inbred) heterotic groups, and two inbreds not related to any of them. The ten lines were used in 45 crosses in a diallel without reciprocal combinations. The experimental design was a randomized complete block design with three replications in three environments. Combining abilities for yield were evaluated after Griffing (1956). Heterosis was estimated as better-parent (BP) heterosis (Kraljević-Balalić et al., 1991).

The proteins were isolated from inbred germs according to Wang et al. (1994) and separated by polyacrylamide gel electrophoresis according to Leammli (1970). The genomic DNA was isolated from inbred germs following the protocol of Saghai-Maroof et al. (1984) and RAPD was performed using the modified protocol of Williams et al. (1990). The amplified bands were scored based on the 1/0 (presence/absence) system. Genetic distances among all possible pairs of inbred lines were estimated from protein and RAPD data according to Nei and Li (1979). Correlations between GD and heterosis, as well as GD/SCA, based both on protein and RAPD markers, were calculated by Spearman's rank correlation coefficient (Zar, 1999).

Results and discussion

ANOVA for combining abilities showed highly significant values for both GCA and SCA. However, the higher value obtained for SCA indicated that dominant gene action had a crucial effect on the inheritance of this trait. Two inbreds (B 97 and ZPPL 204) had highly significant values of SCA, indicating their potential usefulness in the process of breeding for higher yields. Twenty out of 45 F_1 combinations had significant or highly significant values of SCA (Table 1).

Table 1

GCA effects (diagonal) and SCA effects (off diagonal)

Inbreds	ZPPL149	ZPPL148	ZPPL225	ZPPL151	ZPPL204	ZPPL15	ZPPL200	ZPPL80	B97	ZPPL52
ZPPL 149	0.15	0.98	–0.03	1.67	2.41*	–1.13	2.19*	2.44**	0.50	2.12*
ZPPL 148		–0.72*	0.73	1.45	2.23*	1.36	0.96	3.14**	0.23	0.48
ZPPL 225			0.32	3.05**	2.52*	0.14	1.74	1.80*	0.97	1.64
ZPPL 151				–0.33	–0.67	0.96	1.87*	–0.37	1.45	1.94*
ZPPL 204					0.86**	3.61**	–0.45	–0.32	2.40*	1.65
ZPPL 15						0.38	3.00**	2.47*	2.53*	1.95*
ZPPL 200							–0.62*	–0.88	1.99*	2.04*
ZPPL 80								–0.86**	1.99*	1.02
B 97									0.88*	0.67
ZPPL 52										–0.07

Values for heterosis were in general very high and significant. Only a few F_1 combinations did not have significant values for heterosis (data not shown).

The analysis of embryo salt-soluble proteins showed that all studied genotypes had a specific protein pattern. A total of 42 protein fractions of different molecular weight were observed, 76% of which were polymorphic. The shortest GD was found between inbred lines ZPPL 204 and ZPPL 200 (0.094). These lines were of the same origin, with one common parent. Maximum GD (0.359) was observed between two pairs of inbreds: ZPPL 148 and ZPPL 15, and ZPPL 148 and ZPPL 52. These results are in good agreement with the pedigrees of the lines. The inbred ZPPL 148 was derived from Wf 9, ZPPL 15 was obtained by recurrent selection from Iowa Stiff Stalk Synthetic and ZPPL 52 is a European dent line (Table 2).

Based on the results of previous screening of RAPD primers for polymorphism with maize inbred lines, only primers that gave highly reproducible RAPD patterns were used for the present study. The reproducibility of RAPD fragments was tested in two rounds of amplification with all inbreds. Ten random 10-mer primers from Genosys Biotechnologies were used to amplify fragments from the DNA templates of 10 inbreds. A total of 68 RAPD fragments of different molecular weight were scored, 81% of which were polymorphic and gave 3 to 11 fragments per primer. Previous investigations of molecular marker application in genetic studies also showed that RAPD markers reveal a high level of polymorphism in the maize genome (Heun and Helentjaris, 1993).

The GD calculated from 45 combinations of 10 parental lines ranged from 0.124 in the combination ZPPL 15 and ZPPL 149 to 0.674 between the inbreds ZPPL 148 and ZPPL 15, the same combination that had the maximum GD based on protein markers. The combination that was first based on protein markers, had the second value of GD based on RAPD (Table 3).

Table 2

Genetic distance among inbred lines based on protein markers

Inbreds	ZPPL148	ZPPL225	ZPPL151	ZPPL204	ZPPL15	ZPPL200	ZPPL80	B97	ZPPL52
ZPPL149	0.231	0.123	0.228	0.213	0.253	0.238	0.241	0.288	0.220
ZPPL148		0.280	0.255	0.200	0.359	0.273	0.231	0.213	0.359
ZPPL225			0.269	0.254	0.161	0.288	0.286	0.298	0.228
ZPPL151				0.107	0.311	0.167	0.134	0.207	0.214
ZPPL204					0.290	0.094	0.115	0.194	0.258
ZPPL15						0.323	0.322	0.267	0.200
ZPPL200							0.115	0.226	0.311
ZPPL80								0.193	0.236
B97									0.276

Table 3
Genetic distance among inbred lines based on RAPD

Inbreds	ZPPL148	ZPPL225	ZPPL151	ZPPL204	ZPPL15	ZPPL200	ZPPL80	B97	ZPPL52
ZPPL149	0.221	0.198	0.320	0.440	0.124	0.325	0.272	0.289	0.310
ZPPL148		0.260	0.391	0.420	0.674	0.582	0.632	0.160	0.318
ZPPL225			0.528	0.532	0.306	0.381	0.425	0.352	0.372
ZPPL151				0.148	0.438	0.268	0.165	0.330	0.442
ZPPL204					0.561	0.150	0.234	0.432	0.375
ZPPL15						0.613	0.592	0.421	0.512
ZPPL200							0.430	0.398	0.578
ZPPL80								0.415	0.552
B97									0.173

The concurrence between the GD and heterosis, and between GD and SCA was established by Spearman's rank correlation coefficient. Values of the correlations between GD and heterosis and GD/SCA were lower based on protein markers (0.422**, 0.309*, respectively) than those obtained by RAPD. A very strong correlation was found between GD and heterosis (0.876**), while it was also highly significant between GD and SCA (0.671**), based on RAPD. This indicates that there is good agreement between the genetic distance based on RAPD markers and the combining abilities and especially between GD and heterosis, while the protein markers could only be used for preliminary screening and grouping inbreds into heterotic groups without predicting hybrid performances.

The results of this study showed that the estimation of the genetic distance between maize inbred lines by different marker methods is in agreement with data on the origin of the inbreds and their combining abilities, as well as the heterosis obtained in their crosses. While the polymorphism of the protein markers could be used as a preliminary method for the characterization of inbred lines (Wang et al., 1994), RAPD markers detect larger genetic variability and are therefore more suitable for genetic research. Applying the method of RAPD markers, Lanza et al. (1997) reported that genetic divergence based on RAPD markers can be used to establish heterotic groups, though this method was not very efficient in predicting the performance of single crosses.

One of the major objectives in hybrid breeding is the determination of heterotic pools and simplifying the choice of parent lines for the production of high-yielding hybrids (Lübberstedt et al., 2000). Therefore, the molecular marker method offers a reliable and effective means of assessing genetic diversity within and between maize populations (Pejić et al., 1998; Reif et al., 2003). In this way field trials for the identification of promising heterotic patterns can be planned more efficiently based on prior information obtained by molecular markers, which could make a great contribution to the efficiency of maize breeding.

References

Beck, D. L., Vasal, S. K., Carossa, J., (1990): Heterosis and combining ability of CIMMYT's tropical early and intermediate maturity maize (*Zea mays* L.) germplasm. *Maydica*, **35**, 279–285.

Drinić-Mladenović, S., Srdić, J., Drinić, G., Konstantinov, K. (2004): Genetic divergence of maize inbred lines based on molecular markers. pp. 53–56. In: Vollmann, J., Grausgruber, H., Ruckenbauer, P. (eds.), *Proceedings of the 17th EUCARPIA Congress, Genetic Variation for Plant Breeding,* Tulln, Austria. BOKU – University of Natural Resources and Applied Life Sciences, Vienna, Austria.

Drinić-Mladenović, S., Trifunović, S., Drinić, G., Konstantinov, K. (2002): Genetic divergence and its correlation to heterosis in maize as revealed by SSR-based markers. *Maydica*, **47**, 1–8.

Gethi, J. G., Labate, J. A., Lamkey, K. R., Smith, M. E., Kresovich, S. (2002): SSR variation in important U.S. maize inbred lines. *Crop Sci.,* **42**, 952–957.

Griffing, B. (1956): Concept of general and specific combining ability in relation to diallel crossing systems. *Australian J. Biol. Sci.,* **9**, 463–493.

Hahn, V., Blankenhorn, K., Schwall, M., Melchinger, A. E. (1995): Relationship among early European maize inbreds: III. Genetic diversity revealed with RAPD markers and comparison with RFLP and pedigree data. *Maydica*, **40**, 299–310.

Heun, M., Helentjaris, T. (1993): Inheritance of RAPDs in F_1 hybrids in corn. *Theor. Appl. Genet.*, **85,** 961–968.

Kraljević-Balalić, M., Petrović, S., Vapa, Lj. (1991): *Genetika: teorijske osnove sa zadacima.* (Theoretical basis and tasks.) Faculty of Agricultural and Natural-Mathematical Sciences. University of Novi Sad, Serbia. pp. 322–332.

Lanza, L., de Souza, S., Hoboni, L., Vierra, H., de Souza, A. (1997): Genetic distance of inbred lines and prediction of maize single-cross performance using RAPD markers. *Theor. Appl. Genet.*, **94**, 1023–1030.

Leammli, U. K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature*, **227**, 680–685.

Lübberstedt, T., Melchinger, A. E., Dußle, C., Vuylsteke, M., Kuiper, M. (2000): Relationships among early European maize inbreds: IV. Genetic diversity revealed with AFLP markers and comparison with RFLP, RAPD, and pedigree data. *Crop Sci.*, **40**, 783–791.

Melchinger, A. E., Boppenmaier, J., Dhillon, B. S., Pollmer, W. G., Hermann, R. G. (1992): Genetic diversity for RFLPs in European maize inbreds: II. Relation to performance of hybrids within versus between heterotic groups for forage traits. *Theor. Appl. Genet.*, **84**, 672–681.

Nei, M., Li, W. H. (1979): Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. USA*, **76**, 5269–5273.

Pejić, I., Ajmone-Marsan, P., Morgante, M., Kozumplick, V., Castiglioni, P., Taramino, G., Motto, M. (1998): Comparative analysis of genetic similarity among maize inbred lines detected by RFLPs, RAPDs, SSRs and AFLPs. *Theor. Appl. Genet.*, **97**, 1248–1255.

Reif, J. C., Melchinger, A. E., Xia, X. C., Warburton, M. L., Hoisington, D. A., Vasal, S. K., Srinivasan, G., Bohn, M., Frisch, M. (2003): Genetic distance based on simple sequence repeats and heterosis in tropical maize populations. *Crop Sci.,* **43**, 1275–1282.

Saghai-Maroof, M. A., Soliman, K. M., Jorgenson, R. A., Allard, R.W. (1984): Ribosomal DNA spacer-length polymorphism in barley: Mendelian inheritance, chromosomal location and population dynamics. *Proc. Natl. Acad. Sci. USA*, **81**, 8014–8018.

Stojšin, D., Kannenberg, L. W., Rajnpreht, J., Paul, P. K., Stojšin, R. (1996): Genetic relationship among commercial corn hybrids and parents based on RAPD analyses of pericarp and embryo DNA. *Genetika*, **28**, 137–150.

Sun, G. L., William, M. W., Kasha, K. J., Pauls, K. P. (2001): Microsatellite and RAPD polymorphism in Ontario corn hybrids are related to commercial sources and maturity ratings. *Mol. Breed.*, **7**, 13–24.

Wang, C., Bian, K., Zhang, H., Zhou, Z., Wang, J. (1994): Polyacrylamide gel electrophoresis of salt soluble proteins for maize variety identification and genetic purity assessment. *Seed Sci. & Tehnology*, **22**, 51–57.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalsky, J. A., Tingey, S. V. (1990): DNA polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucleic Acid Res.*, **18**, 6531–6535.

Zar, J. H. (1999): *Biostatistical Analysis*, 4th edition. Prentice-Hall, Inc., New Jersey, USA.

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HYBRID MAIZE BREEDING WITH DOUBLED HAPLOIDS: COMPARISON BETWEEN SELECTION CRITERIA

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The optimum allocation of breeding resources is crucial for the efficiency of breeding programmes. The objectives were to (i) compare selection gain ΔG_k for finite and infinite sample sizes, (ii) compare ΔG_k and the probability of identifying superior hybrids (P_k), and (iii) determine the optimum allocation of the number of hybrids and test locations in hybrid maize breeding using doubled haploids. Infinite compared to finite sample sizes led to almost identical optimum allocation of test resources, but to an inflation of ΔG_k. This inflation decreased as the budget and the number of finally selected hybrids increased. A reasonable P_k was reached for hybrids belonging to the $q = 1\%$ best of the population. The optimum allocations for $P_k(q)$ and ΔG_k were similar, indicating that $P_k(q)$ is promising for optimizing breeding programmes.

Keywords: optimum allocation, selection gain, finite and infinite sample size, probability

Introduction

The optimum allocation of financial and breeding resources is of fundamental importance for the efficiency of breeding programmes. Currently, doubled haploids (DHs) are adopted as a routine method in commercial maize breeding programmes (Seitz, 2005). Their efficient use requires the optimization of the entire breeding strategy in order to maximize progress from selection. To quantify the progress from k selection stages, various criteria have been used. Selection gain ΔG_k is the most widely used criterion to optimize selection processes. The selection theory for ΔG_k was developed assuming an infinite sample size, although populations of medium size are commonly used in plant breeding (Cochran, 1951; Hanson and Brim, 1963; Utz, 1969; Grüneberg et al., 2004). This assumption simplifies the calculations considerably. Inflated ΔG_k and a slightly different optimum allocation of test resources for infinite compared to finite sample size were reported in the literature (Cochran, 1951; Finney, 1966; Utz, 1969; Young, 1976). However, these studies were conducted more than 30 years ago and the limited computing power available at that time restricted the accuracy of the simulations. The probability of identifying superior genotypes (P_k) represents an interesting alternative to ΔG_k for optimizing the allocation of test resources (Robson et al., 1967; Johnson, 1989; Longin et al., 2006).

The allocation of test resources in hybrid maize breeding with DHs was optimized under one- and two-stage selection for testcross performance with a given tester by using Monte Carlo simulations and numerical integration. The objectives were to (i) compare ΔG_k for finite and infinite sample sizes, (ii) compare ΔG_k and P_k, and (iii) determine the optimum allocation of the number of hybrids and test locations.

Materials and methods

Selection strategies

A total of N_1 hybrids generated by crossing DH lines to a given tester are available each year to start selection. The tester can be any population with an arbitrary structure, such as an inbred line, single cross, or random mating population. The N_f phenotypically best hybrids are finally selected. A value of $N_f = 1$ was assumed to emphasize the interest in the very best hybrid. The target variable is the genotypic value of testcross performance with a given tester for a certain trait or index of traits. With one-stage selection, selection is based on field tests in a single year. With two-stage selection, field tests are conducted in two years, with a subset of the most superior hybrids N_2 selected after the first year being evaluated in the second year. At stage j (j = 1, 2), selection among N_j hybrids is based on the phenotypic mean of testcross performance at this stage with a given tester evaluated in L_j locations with R_j replications. Without an upper limit on L_j, R_j = 1 is optimal for ΔG_k (Sprague and Federer, 1951; Utz, 1969). The R_j value was thus set to 1.

Economic frame and quantitative genetic parameters

A fixed total budget B for (i) producing the DH lines and (ii) evaluating their testcross progenies in two selection stages was defined in terms of testcross plot equivalents as $B = N_1C + N_1L_1R_1 + N_2L_2R_2$ assuming equal plot sizes in both selection stages. Therein, the production cost C of one DH line was assumed to equal half the cost of one field plot ($C = 0.5$), corresponding to the actual costs of DH production in breeding companies most advanced in the DH technique (Seitz, pers. comm.). The focus was generally on a budget of B = 20 000 field plot equivalents. Three ratios of variance components ($\sigma_g^2 : \sigma_{gl}^2 : \sigma_{gy}^2 : \sigma_{gly}^2 : \sigma_e^2$) were considered, where σ_g^2 refers to the genotypic variance, σ_{gl}^2 to the variance of genotype × location interactions, σ_{gy}^2 to the variance of genotype × year interactions, σ_{gly}^2 to the variance of genotype × location × year interactions, and σ_e^2 to the error variance. Values were set to $VC1 = 1 : 0.25 : 0.25 : 0.5 : 1$, $VC2 = 1 : 0.5 : 0.5 : 1 : 2$ and $VC3 = 1 : 1 : 1 : 2 : 4$, resulting in a heritability on a one-plot basis of 0.33, 0.20 and 0.11, respectively. These ratios were chosen based on combined analyses of variance of testcrosses of DH populations from commercial breeding programmes (Longin et al., 2006).

Calculation of optimization criteria

Selection gain for finite sample size ($\Delta\hat{G}_k$) and the probability of identifying superior hybrids ($\hat{P}_k$) were estimated by Monte Carlo simulations according to Longin et al. (2006) assuming a standard normal distribution of the hybrids in a whole breeding programme. The calculation of selection gain for infinite sample sizes ($\Delta G(\text{inf})_k$) with numerical integration is based on uni- and bivariate normal integrals for selected fractions $\alpha_j = N_{j+1}/N_j$ and the square root of heritability of phenotypic means at stage j (*cf.* Cochran, 1951). An admissible allocation of test resources refers to tuples $(N_j\,;\,L_j)$ for all stages j. An element $(N^*_j\,;\,L^*_j)$ is denoted as an optimum allocation if it maximizes the optimization criterion in the set of admissible allocations. The values of each optimization criterion at its corresponding optimum allocation $(N^*_j\,;\,L^*_j)$ were denoted as $\Delta\hat{G}^*_k$ and $\hat{P}^*_k(q)$ for the Monte Carlo simulations and $\Delta G(\mathrm{inf})^*_k$ for the numerical integration. The optimization criteria $\Delta\hat{G}_k$ and $\hat{P}_k(q)$ are estimated with a precision of 0.01 to limit the number of simulation runs to a manageable number (Longin et al., 2006). Thus, the optimum allocation $(N^*_j\,;\,L^*_j)$ was determined following Utz (1969) such that the number of locations was minimum among all allocations within a 0.01 drop-off of all optimization criteria, since breeders prefer tests in fewer locations for technical reasons if this only affects the optimization criteria marginally.

Results

With increasing L_1, the optimization criteria $\Delta G(\mathrm{inf})_1$, $\Delta\hat{G}_1$ and $\hat{P}_1(q)$ increased up to an optimum and decreased slightly thereafter (Fig. 1). The increase in $\Delta G(\mathrm{inf})_1$, $\Delta\hat{G}_1$ and $\hat{P}_1(q)$ was largest between $L_1 = 1$ and $L_1 = 6$. All response curves were flat in the vicinity of the maximum. With decreasing q, the slope of $\hat{P}_1(q)$ decreased. The optimum allocation and the standard deviations (*SD*s) of the optimization criteria were similar for $\Delta G(\mathrm{inf})^*_1$ and $\Delta\hat{G}^*_1$ (Table 1). Differences were observed for $\Delta G(\mathrm{inf})^*_1$ compared to $\Delta\hat{G}^*_1$. With increasing N_f, the ratio $\Delta G(\mathrm{inf})^*_1/\Delta\hat{G}^*_1$ decreased from 8.5 to 2.9% for $B = 200$ and from 3.2 to 0.5% for $B = 20\,000$. With increasing B, the ratio $\Delta G(\mathrm{inf})^*_1/\Delta\hat{G}^*_1$ decreased from 8.5% to 3.2% for $N_f = 1$ and from 2.9 to 0.5% for $N_f = 20$. The optimum allocation of test resources based on the same *VC* but different optimization criteria differed largely for small values of q ($q = 0.1$; 0.01%) and large non-genetic variance (*VC*3, Table 2). For instance, for *VC*3, the optimum number of hybrids N^*_j was approximately doubled and the optimum number of locations L^*_j was more than halved for $\hat{P}^*_k$ (0.01%) in comparison with $\Delta\hat{G}^*_1$.

The optimum number of initial lines N^*_1 and test locations for two-stage selection was about twice as large as for one-stage selection (Table 2). This was due to the optimum allocation of two-stage selection, which comprised a large number of initial hybrids N^*_1 tested in a small number of test locations L^*_1 the first stage, and a small number of selected hybrids N^*_2 tested in a large number of test locations L^*_2 the second stage. Furthermore, values of $\Delta\hat{G}^*_k$ and of $\hat{P}^*_k(1\%)$, $\hat{P}^*_k(0.1\%)$, and $\hat{P}^*_k(0.01\%)$ were 18%, 40%, 100% and 250%, respectively, higher on average than for one-stage selection.

Discussion

Comparison of selection gain for infinite vs. *finite sample size*

The optimum allocation of test resources regarding selection gain for infinite ($\Delta G(\mathrm{inf})_k^*$) *vs.* finite sample size ($\Delta\hat{G}_k^*$) was almost identical for all the scenarios considered (Table 1). This is in accordance with a previous study (Utz, 1969) and can be explained by the similar response curves for $\Delta G(\mathrm{inf})_k$ and $\Delta\hat{G}_k$ as a function of the number of locations (Fig. 1). The similar response curves are due to the similar slopes of the selection intensity for infinite and finite sample sizes and to the fact that heritability is not affected by the sample size of the population.

For small budgets and number of finally selected lines, $\Delta G(\mathrm{inf})_k^*$ was clearly inflated in comparison to $\Delta\hat{G}_k^*$ (Table 1), which is in harmony with results reported in the literature (Utz, 1969). The inflation of $\Delta G(\mathrm{inf})_k^*$ decreased with increasing B and/or N_f. This can be explained by the distribution of the hybrids. With increasing population size (increasing B), the deviation of the actual distribution for finite sample sizes from the expected standard normal distribution for infinite sample sizes decreases. The impact of N_f can be explained by the fact that the deviation from the standard normal distribution for small sample sizes mainly affects the tails of the distribution.

Table 1

Optimum allocation of test resources maximizing selection gain for infinite ($\Delta G(\mathrm{inf})_1^*$) and finite sample size ($\Delta\hat{G}_1^*$) and their standard deviation (*SD*) for one-stage selection assuming a ratio of variance components of 1 : 0.5 : 0.5 : 1 : 2.

Assumptions		Optimum allocation		*OC*	*SD*[a]
B	N_f	N_1^*	L_1^*		
$\Delta G(\mathrm{inf})_1^*$					
200	1	44	4	1.54	0.83
	5	57	3	1.11	0.39
	20	80	2	0.71	0.21
20 000	1	1904	10	2.60	0.73
	5	2352	8	2.26	0.34
	20	3076	6	1.95	0.18
$\Delta\hat{G}_1^*$					
200	1	44	4	1.42	0.80
	5	57	3	1.08	0.36
	20	133	1	0.69	0.21
20 000	1	1739	11	2.52	0.72
	5	2352	8	2.25	0.34
	20	3076	6	1.94	0.17

[a] Approximated for infinite sample size after Burrows (1975); B = budget in field plot equivalents, N_f = number of finally selected hybrids, N_l^*, L_l^* = optimum number of hybrids and test locations, OC = optimization criterion

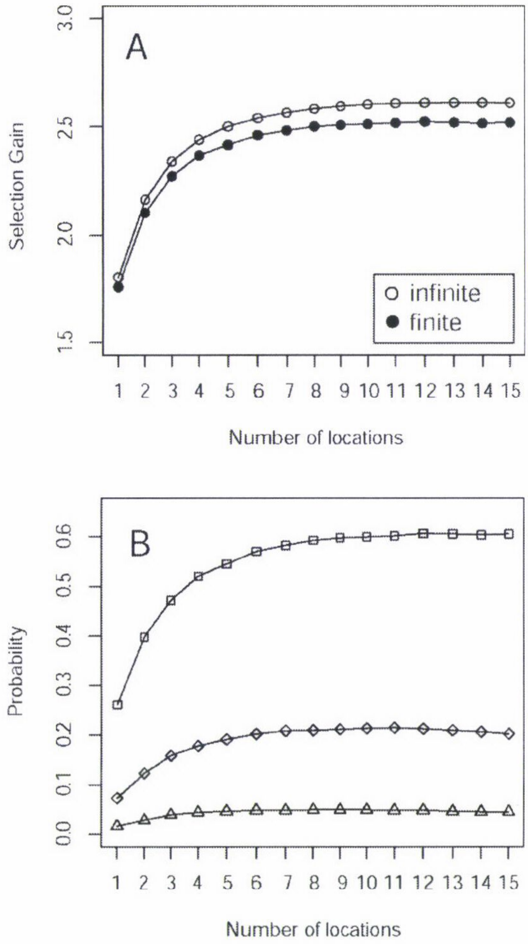

Fig. 1. (A) Selection gain for infinite $\Delta G(\text{inf})_1$ and finite sample size $\Delta\hat{G}_1$ and (B) probability $\hat{P}^*_1(q)$ of identifying one hybrid with a genotypic value belonging to the 1% (□), 0.1% (◊) and 0.01% (Δ) best genotypes of the population as a function of the number of locations for one-stage selection, assuming a budget of 20 000 field plot equivalents and a ratio of variance components of 1 : 0.5 : 0.5 : 1 : 2.

Comparison between several breeding alternatives is normally based on the use of either $\Delta G(\text{inf})_k$ or $\Delta\hat{G}_k$. Thus, the alternatives are equally affected by the inflation of $\Delta G(\text{inf})_k$. For comparison between different B and N_f values, the small bias caused by $\Delta G(\text{inf})_k$ can be neglected in comparison to the large impact of B and N_f on selection gain (Table 1). Consequently, the simplifying assumption of infinite sample sizes for determining the optimum allocation of test resources is justifiable as long as a reduction in computing time and effort is warranted.

Comparison of selection gain with probability of identifying superior hybrids

The optimum allocation of test resources differed for $\Delta\hat{G}^*_k$ and $\hat{P}^*_k(q)$, especially for small values of q and large non-genetic variance (Table 2). For one-stage selection, the closest agreement between the optimum allocation of test resources maximizing $\hat{P}^*_k(q)$, and $\Delta\hat{G}_k$ was observed for q = 5% (data not shown). With decreasing values of q, an increased N^*_1 and a decreased L^*_1 were observed. This can be explained by the fact that the probability that genotypes belonging to the q% best genotypes of the population are among the initial hybrids decreases rapidly with decreasing N_1 and q (Longin et al., 2006). In addition, the slope of the response curves of $\hat{P}_k(q)$ decreased with smaller q (Fig. 1), favouring allocations with smaller L_1.

For two-stage selection, the optimum allocation of test resources maximizing $\hat{P}_k(q)$ and $\Delta\hat{G}_k$ was only comparable for $VC1$ and $q = 0.1\%$. For large non-genetic variance and small q, an increased N^*_1 and a decreased L^*_2 were observed. However, for $q = 1\%$, $VC1$ and $VC2$, a decreased N^*_j and increased L^*_1 were observed in comparison to $\Delta\hat{G}^*_k$ This may be due to the considerably increased N^*_1 in two-stage selection compared to one-stage selection and the consequent increase in the importance of heritability. Nevertheless, values of $\hat{P}_k(q)$ differed only slightly from values of $\hat{P}_k(q)$ at the optimum allocation of test resources with regard to $\Delta\hat{G}^*_k$ (Longin et al., 2006), which can be explained by the flat response curves of $\Delta\hat{G}_k$ and $\hat{P}_k(q)$ in the vicinity of the maximum (Fig. 1).

To have a realistic chance of identifying a superior genotype, $P_k(q)$ should be greater than 75%, permitting only q values of about 1% even for the large budget considered. The choice of the optimization criterion for these q values is not crucial, because the optimum allocation of test resources differed only slightly from those obtained by applying $\Delta\hat{G}_k$. Therefore, the use of $P_k(q)$ seems appealing for the optimization of breeding programmes, favouring a reduction in the number of test locations with a parallel increase in the number of initial hybrids for the selection of very outstanding hybrids.

Two-stage selection – promising method to increase $P_k(q)$

The possibilities of increasing $P_k(q)$ are limited, especially for small values of q (Longin et al., 2006). An increasing budget increases $\hat{P}^*_k(q)$, but the return from investment is rather low. Increasing the number of selection stages from one to two considerably increased $\Delta\hat{G}^*_k$ and $\hat{P}^*_k(q)$ (Table 2). This is due to the optimum allocation of test resources in two-stage selection involving the evaluation of (i) a large number of hybrids in a small number of test locations in the first year and (ii) a small number of selected superior hybrids in a large number of test locations in the second year. $\hat{P}^*_k(q)$ for small values of q was particularly improved by two-stage selection in comparison to one-stage selection, which may mainly be due to the increased N^*_1. With one-stage selection, breeders could exploit the progress of selection by improved hybrids one year earlier. However, the limited possibilities for increasing $P_k(q)$ make the use of two-stage instead of one-stage selection very appealing to identify hybrids with outstanding performance.

Table 2

Optimum allocation of test resources maximizing selection gain $\Delta\hat{G}_k^*$ or probability $\hat{P}_k^*(q)$ of identifying one hybrid with a genotypic value belonging to the $q = 1$, 0.1 and 0.01% best genotypes in the population assuming a budget of 20 000 field plot equivalents. (k = number of selection stages, VC = ratio of variance components, N_j^*, L_j^* = optimum number of hybrids and test locations at stage j, OC = optimization criterion, SD = standard deviation estimates among runs)

Assumptions		Optimum allocation					
k	VC	N_1^*	N_2^*	L_1^*	L_2^*	OC	SD
$\Delta\hat{G}_k^*$							
1	1[a]	2666	–	7	–	2.87	0.63
1	2[b]	1739	–	11	–	2.25	0.72
1	3[c]	1481	–	13	–	2.11	0.81
2	1	10440	217	1	20	3.33	0.56
2	2	6090	129	2	37	2.99	0.64
2	3	3660	84	4	42	2.57	0.73
$\hat{P}_k^*(1\%)$							
1	1	2666	–	7	–	0.81	0.40
1	2	2105	–	9	–	0.60	0.49
1	3	1739	–	11	–	0.39	0.49
2	1	7224	102	2	19	0.97	0.19
2	2	4926	89	3	31	0.85	0.36
2	3	3967	58	4	37	0.63	0.48
$\hat{P}_k^*(0.1\%)$							
1	1	3636	–	5	–	0.35	0.48
1	2	2666	–	7	–	0.21	0.41
1	3	2666	–	7	–	0.10	0.30
2	1	10440	217	1	20	0.66	0.47
2	2	6627	143	2	24	0.43	0.50
2	3	5001	96	3	26	0.23	0.42
$\hat{P}_k^*(0.01\%)$							
1	1	5714	–	3	–	0.09	0.08
1	2	4444	–	4	–	0.04	0.04
1	3	5714	–	3	–	0.01	0.01
2	1	10750	298	1	13	0.23	0.42
2	2	7104	140	2	16	0.12	0.33
2	3	5450	66	3	14	0.05	0.23

[a] VC1 = 1: 0.25 : 0.25 : 0.5 : 1; [b] VC2 = 1: 0.5 : 0.5 : 1 : 2; [c] VC3 = 1 : 1 : 1 : 2 : 4

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References

Burrows, P. M. (1975): Variances of selection differentials in normal samples. *Biometrics*, **31**, 125–133.

Cochran, W. G. (1951): Improvement by means of selection. *2nd Berkeley Symp. Math. Stat. Prob.*, pp. 449–470.

Finney, D. J. (1966): An experimental study of certain screening processes. *J. Roy. Stat. Soc. B.*, **28**, 88–109

Grüneberg, W. J., Abidin, E., Ndolo, P., Pereira, C. A., Hermann, M. (2004): Variance component estimations and allocation of resources for breeding sweetpotato under East African conditions. *Plant Breeding*, **123**, 311–315.

Hanson, W. D., Brim, C. A. (1963): Optimum allocation of test material for two-stage testing with an application to evaluation of soybean lines. *Crop Sci.*, **3**, 43–49.

Johnson, B. (1989): The probability of selecting genetically superior S_2 lines from a maize population. *Maydica*, **34**, 5–14.

Longin, C. F. H., Utz, H. F., Reif, J. C., Schipprack, W., Melchinger, A. E. (2006): Hybrid maize breeding with doubled haploids: I. One-stage versus two-stage selection for testcross performance. *Theor. Appl. Genet.*, **112**, 903–912.

Robson, D. S., Powers, L., Urquhart, N. S. (1967): The proportion of genetic deviates in the tails of a normal population. *Theor. Appl. Genet.*, **37**, 205–216.

Seitz, G. (2005): The use of doubled haploids in corn breeding. In: *Proc. of the 41st Annual Illinois Corn Breeders' School 2005*, Urbana-Champaign, Illinois, USA, p. 18.

Sprague, G. F., Federer, W. T. (1951): A comparison of variance components in corn yield trials: II. Error, year × variety, location × variety and variety components. *Agron. Jour.*, **42**, 535–541.

Utz, H. F. (1969): Multi-stage selection in plant breeding (In German). *Arbeiten der Universität Hohenheim, Vol. 49*. Verlag Eugen Ulmer, Stuttgart, Germany.

Young, J. C. (1976): Varietal screening from finite normal populations. *J. Am. Stat. Assoc.*, **71**, 87–92.

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IMPROVEMENT OF EFFECTIVENESS IN MAIZE BREEDING

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Plant regeneration via tissue culture is becoming increasingly more common in monocots such as maize (*Zea mays* L.). Pollen (gametophytic) selection for resistance to aflatoxin in maize can greatly facilitate recurrent selection and the screening of germplasm for resistance at much less cost and in a shorter time than field testing. *In vivo* and *in vitro* techniques have been integrated in maize breeding programmes to obtain desirable agronomic attributes, enhance the genes responsible for them and speed up the breeding process. The efficiency of anther and tissue cultures in maize and wheat has reached the stage where they can be used in breeding programmes to some extent and many new cultivars produced by genetic manipulation have now reached the market.

Key words: *Zea mays*, callus induction, aflatoxin, grain yield

Introduction

The regeneration of plants from tissue cultures was first reported in maize (*Zea mays* L.) by Green and Philips (1975), who utilised immature embryos as the tissue source. Using the same tissue system, Springer et al. (1979) demonstrated that plant regeneration took place by means of organogenesis.

Rice et al. (1978) found that plant regeneration could also occur by somatic embryogenesis. Both types of regeneration arise from hard, white or yellow callus, which can be clearly distinguished from the granular, greyish-yellow, translucent callus that is incapable of plant regeneration. The regeneration of maize from cell and tissue cultures has been limited to a few specific genotype and medium combinations (Green and Philips, 1975; Hodges et al., 1985; Kamo et al., 1985). Medium improvements boosted the average regeneration, but genotypic differences remained (Duncan et al., 1985). Studies on the genotype component can be used to predict probabilities for a desired response level and to describe in more detail the nature of the tissue culture response (Tomes and Smith, 1985).

Mycotoxins, in general, reportedly contaminate about one-quarter of the world's yearly food and feed crops. The effects of mycotoxin consumption on animal health range from decreased growth rates and reproductive efficiency to mortality. In addition, there is increasing concern about the effects of aflatoxin on human health, as aflatoxins have been linked to liver cancer. The occurrence of aflatoxin in food is viewed as a potential threat to the food supply. It has been deemed necessary to develop efficient methods that will prevent aflatoxin contamination in crops. Host plant resistance is the most logical and useful method of control (Dickson et al., 1992; Kang et al., 1992).

Thus far, only a few studies on the inheritance of resistance have been conducted (Widstrom et al., 1984; Gardner et al., 1987; Darrah et al., 1987; Gorman and Kang, 1991; Pepó and Szabó, 1998; Tóth and Bódi, 2006). Progress in elucidating genetic mechanisms and identifying sources of resistance to aflatoxin has been slow, primarily because genotype evaluation in the field (sporophytic selection) is laborious, expensive and time-consuming. Selection at the pollen level (male gametophytic selection) to screen for resistance to aflatoxin did not receive much attention until recently. The gametophytic generation has appropriately been called 'the forgotten generation'.

A total of 60–70% of structural genes controlling traits in the sporophytic generation (plant) are expressed in the gametophytic generation (pollen) (Mulcahy, 1971; Mulcahy and Mulcahy, 1987; Ottaviano et al., 1980; Tanksley et al., 1981; Smith, 1986). This genetic overlap between the sporophytic and gametophytic generations offers a tremendous potential for modifying the sporophyte by applying selection pressure on the gametophyte. A maize plant produces 2 to 5 million pollen grains that can be subjected to selection. Selection pressure applied to pollen produced by a genetically homogeneous, heterozygous plant is expected to produce genetic changes in the sporophytic population (Ottaviano and Mulcahy, 1989).

Intergeneric somatic hybridization was performed between albino maize (*Zea mays* L.) protoplasts and mesophyll protoplasts of wheat (*Triticum aestivum* L.) by means of PEG treatments. None of the parental protoplasts were able to produce green plants without fusion (Mórocz et al., 1993).

Materials and methods

Two maize populations developed in the Louisiana State University maize breeding programme, viz. (Mo17 × B73) × Yellow Creole and (Mo17 × B 73) × (L331 × Yellow Creole), hereafter referred to as population L91R and population L331, respectively, were evaluated for their *in vitro* culturability and regeneration potential. The S_0, S_1 and S_2 generations were subjected to tissue culturing using the following procedure. The seeds were surface-sterilised for 10 min in 0.2% aqueous mercurous chloride solution, rinsed overnight under running tap water and re-sterilised for 5 min in 0.2% aqueous mercurous chloride solution, followed by several water rinses. Twenty-five kernels of each generation were germinated. Aseptic seedlings were grown on a 1% agar-solidified medium containing the inorganic constituents of Murashige and Skoog (1962): 3% sucrose, 26.7 μM glycine, 4.1 μM nicotinic acid, 2.4 μM pyridoxine-HCl and 0.3 μM thiamine-HCl. N6 medium was used with 100 μmol Fe-EDTA concentration to initiate anther culture. A saccharose concentration of 10% supplemented with activated charcoal was applied to induce calli in maize.

The radicles were aseptically separated from the plumules at the scutellar node and the explants were cut into five pieces each 2–3 mm long. These explants and intact, mature embryos were plated on MS medium. The pH was adjusted to 5.8 before autoclaving. Incubation was done at 26°C with a 16/8 h photoperiod.

The 2,4-D concentration was 2.5 mg L^{-1}. After callus induction, meristematic segments were discarded; the remainder were transferred to the above culture medium for callus proliferation. To induce further differentiation, the calli were subcultured on MS medium supplemented with different concentrations of 2,4-D and zeatin. Regenerated plantlets were transferred to hormone-free medium for root development.

Anthers with mid-uninucleate microspores were cold-treated at 8–10°C for 14 days and heat-preincubated in the dark at 27±2°C for 7 days. To increase the frequency of embryoids the medium was supplemented with activated charcoal and 0.1 mg L^{-1} TIBA. The embryoids were further cultured on N6 and M9 media to induce differentiation.

In the breeding programme, diallels were obtained by crossing four maize inbred lines (P2, P34, P50 and P61). The general and specific combining abilities for callus weight and grain yield were estimated using a full diallel system according to a modified version of Griffing's Method 1.

Results

It can be seen from Table 1 that the callus induction frequency for the two populations ranged from 4.7 to 60.9%. The highest frequency of callus formation was exhibited by radicle tissue and the lowest by the embryo. In both populations, the callus induction frequency decreased as the level of homozygosity increased, which suggested that callus induction was controlled primarily by dominant gene action.

Table 1

Callus induction and regeneration for populations L331 and L91R

Explant	Inbred stage	Callus induction		Regeneration	
		L331	L91R	L331	L91R
Radicle (R)	S_o	60.9	41.1	4.75	1.51
	S_1	58.0	32.0	4.45	0.0
	S_2	56.0	23.9	0.0	0.0
	Mean	58.3	33.4	2.70	0.59
Plumule (P)	S_o	41.9	30.1	9.94	3.05
	S_1	39.0	23.9	6.65	1.26
	S_2	38.9	19.0	4.43	0.0
	Mean	40.0	25.1	6.95	1.65
Embryo (E)	S_o	40.0	9.8	5.00	2.17
	S_1	38.2	7.2	2.06	1.20
	S_2	35.8	4.7	1.05	0.0
	Mean	38.0	7.5	2.75	1.25
Mean: R+P+E	S_o	50.9 (601/1181)	33.3 (228/1016)	7.03 (83/1181)	2.26 (23/1016)
	S_1	47.9 (590/1232)	26.1 (233/893)	4.79 (59/1232)	0.67 (6/893)
	S_2	46.9 (577/1230)	19.9 (134/673)	3.92 (25/1230)	0.0 (0/673)

The explants had different plant regeneration percentages in both populations. The maximum number of plants was regenerated from plumules in the L331 population. No plant regeneration was noted in the S_2 generation of the L91R population. The results suggested that plant regeneration would be increasingly more difficult in the inbred generations and that prior to embarking on a tissue culture-based breeding programme, responsive genotypes should be identified.

Microscopic investigations were carried out to investigate the effect of *Aspergillus flavus* spores and the aflatoxin B1 on *in vitro* pollen germination and it was demonstrated that both inhibited pollen germination (Table 2).

Table 2

Mean percentage germination of pollen grains from the F_2 of the single cross (Mp 313 E× SC 212 M)

Treatment	Mean pollen grain germination (%)
Control	71.5 a*
Aspergillus parasiticus spores	44.2 b
Aflatoxin B1 (400 ppm)	42.4 b
Aspergillus flavus spores	23.7 c

Means followed by the same letter are not significantly different at the 5% level of probability according to Duncan's New Multiple Range Test.

The callus induction and yielding ability of four maize inbred lines (P2, P34, P50 and P61) were compared in a diallel system. The general and specific combining abilities for callus weight and grain yield were estimated (Tables 3, 4, 5 and 6) using a full diallel system according to a modified version of Griffing's Method 1.

Table 3

Values of general combining ability (GCA) for callus weight

Inbred lines	GCA [g]
P2	0.1110
P34	–0.0840
P50	–0.7545
P61*	0.7180

*Registered maize line

Table 4

Values of general combining ability (GCA) for grain yield

Inbred lines	GCA [t/ha]
P2	–0.384
P34	–0.093
P50	–0.073
P61* (3)	0.550

*Registered maize line

Table 5
Results of callus induction in diallel analysis

Female lines	Male lines			
	P2	P34	P50	P61
P2	–4.438 (SCA)	1.858 (SCA)	1.719 (SCA)	0.861 (SCA)
P34	–2.250 (RE)	–2.988 (SCA)	0.024 (SCA)	1.106 (SCA)
P50	–2.080 (RE)	–0.500 (RE)	–5.825 (SCA)	4.083 (SCA)
P61	–1.995 (RE)	–0.955 (RE)	–0.720 (RE)	–6.050 (SCA)

SCA = specific combining ability (g); RE = Effect of reciprocal (g); *LSD$_{5\%}$ = 0.237; R^2 = 0.898

Table 6
Results of grain yield in diallel analysis

Female lines	Male lines			
	P2	P34	P50	P61
P2	–5.333 (SCA)	2.406 (SCA)	1.137 (SCA)	1.789 (SCA)
P34	–0.455 (RE)	–4.436 (SCA)	0.657 (SCA)	1.373 (SCA)
P50	–1.108 (RE)	0.109 (RE)	–4.059 (SCA)	2.265 (SCA)
P61	–1.114 (RE)	–0.260 (RE)	–1.361 (RE)	–5.426 (SCA)

SCA = specific combining ability (t/ha); RE = Effect of reciprocal (t/ha); *LSD$_{5\%}$ (3) = 0.312; R^2 = 0.887

Callus growth was found to be genotype-dependent. The hybrid P61 × P50 had the highest specific combining ability. The highest GCA values for callus weight and grain yield were found for line P61.

The relationship between the callus induction and yield of maize lines and hybrids can be seen in Figure 1.

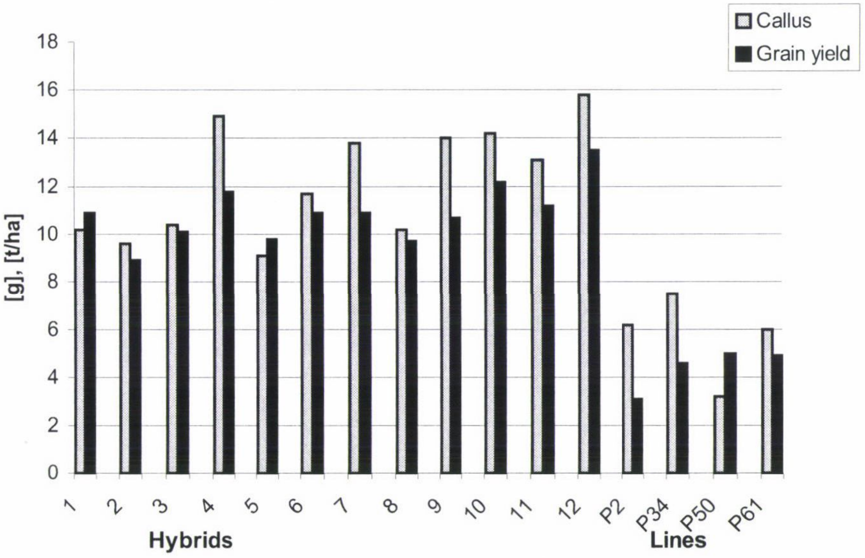

Fig. 1. Relation between the callus induction (g) and yield (t/ha) of maize lines and hybrids

It can be concluded from the results that the genetic abilities of the parents and their hybrids are also manifested under *in vitro* conditions. The determination of the general/specific combining ability and callus growth potential of genotypes could provide important information for field breeding programmes.

Discussion

A wide variety of diploid and doubled haploid lines produced *in vitro* are increasingly used in practical breeding programmes (Fig. 2).

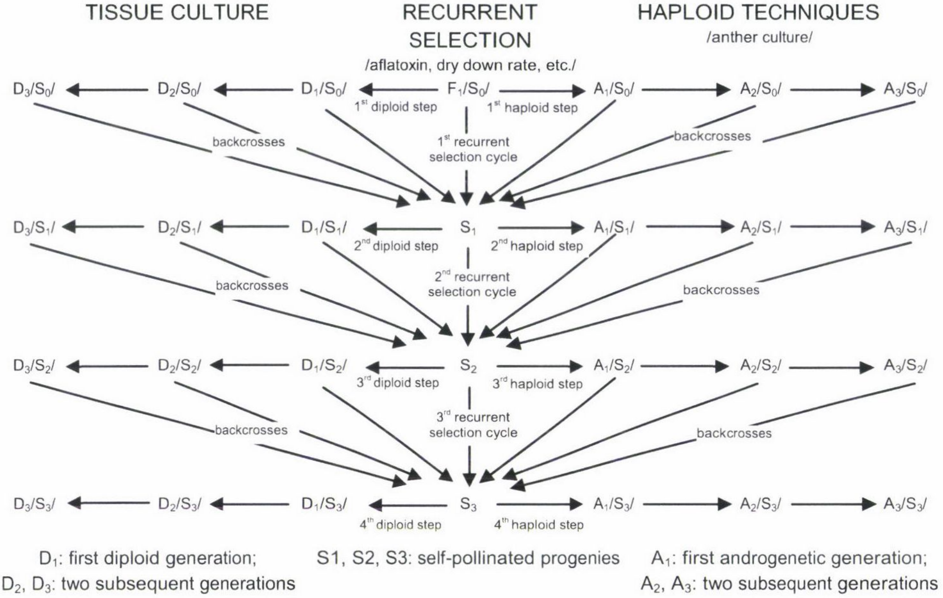

Fig. 2. Integrated maize breedig programme. Starting material: synthetics, hybrids e.g.: [(L697 × Yellow Creole) × (Mo18W × Mp313E)]; De 2205 SC (61 × 50)

Anther culture and tissue culture techniques were employed to develop lines with resistance to aflatoxins and herbicides, and with other desirable agronomic traits such as fast dry-down rate, better stalk and seed quality, and weevil resistance. As seen in Figure 2, a complex maize breeding programme was developed to obtain desirable agronomic attributes, enhance the genes responsible for them and speed up the breeding process. Depending on the nature of the source material, e.g. synthetics, F_1 or open-pollinated varieties, and the breeding aims, one or more haploid (pollen) or diploid (tissue) steps were included and the F_1 hybrids or selfed progenies in later generations served as the source material for haploidization or tissue culture. One haploid step followed by selection in the greenhouse/field or a diploid step after selection at the cell/plant level during the first androgenetic generation (A_1) or diploid progenies (D_1) and two subsequent selfed generations (A_2, A_3, D_2, D_3) proved to be the most efficient procedure, if characters from related varieties were to be combined. To make this genetic manipulation system more efficient, it was combined with several backcrosses.

A combination of conventional and new genetic recombination methods (*in vivo* and *in vitro* genetic manipulation) may result in the development of cereal varieties and hybrids that are better able to meet production demands. The efficiency of anther and tissue cultures in most cereals such as maize has reached the stage where it can be used in breeding programmes to some extent and many new cultivars produced using this system have now reached the market.

References

Darrah, L. L., Lillehoj, E. B., Zuber, M. S., Scott, G. E., Thompson, D., West, D. R., Widstrom, N. W., Fortnum, B. A. (1987): Inheritance of aflatoxin B1 levels in maize kernels under modified natural inoculation with *Aspergillus flavus*. *Crop Sci.*, **27**, 869–872.

Dickson, J. I., Dronovalli, S., Pepo, P., Kondapi, N., Kang, M. S. (1992): Effect of MSMA on *in vitro* corn pollen germination. Southern Branch of American Society of Agronomy, Lexingston, Kentucky. *Agronomy Abstracts*, pp. 45–46.

Duncan, D. R., Williams, E. M., Zehr, B. E., Widholm, J. M. (1985): The production of callus capable of plant regeneration from immature embryos of numerous *Zea mays* genotypes. *Planta,* **165**, 322–332.

Gardner, C. A. C., Darrah, L. L., Zuber, M. S., Wallin, J. R. (1987): Genetic control of aflatoxin production in maize. *Plant Dis*., **71,** 426–429.

Gorman, D. P., Kang, M. S. (1991): Preharvest aflatoxin contamination in maize: Resistance and genetics. *Plant Breeding*, **107,** 1–10.

Green, C. E., Philips, R. L. (1975): Plant regeneration from tissue culture of maize. *Crop Sci.*, **15**, 417–421.

Hodges, T. K., Kamo, K. K., Becwar, R. M., Schroll, S. (1985): Regeneration of maize. pp. 15–33. In: Zaitlin, M., Day, P., Hollaender, A. (eds.), *Biotechnology in Plant Science: Relevance to Agriculture in the Nineteen Eighties*. Academic Press, Orlando, Florida.

Kamo, K. K., Becwar, M. R., Hodges, T. K. (1985): Regeneration of *Zea mays* L. from embryogenic callus. *Bot. Gaz.,* **146**, 327–334.

Kang, M. S., Pepó, P., Gorman, D. P., Dronovalli, S., Dickson, J. I., Kondapi, N. (1992): Corn breeding and genetic investigations. *Report of Projects for 1991*, Dept. of Agronomy, LSU. pp. 71–78.

Mórocz, S., Dudits, D., Golovkin, M. V., Ábrahám, M., Bottka, S., Fehér, A. (1993): Production of transgenic maize plants by direct DNA uptake into embryogenic protoplasts. *Plant Science,* **90**, 41–52.

Mulcahy, D. L. (1971): Correlation between gametophytic and sporophytic characteristics in *Zea mays* L. *Science,* **171**, 1155–1156.

Mulcahy, D. L., Mulcahy, G. B. (1987): The effects of pollen competition. *Am. Scientist*, **75**, 44–50.

Murashige, T., Skoog, F. (1962): A revised media for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant*., **15**, 473–479.

Ottaviano, E., Sari-Gorla, M., Mulcahy, D. L. (1980): Pollen tube growth rates in *Zea mays*: Implications for genetic improvement of crops. *Science*, **210**, 437–438.

Ottaviano, E., Mulcahy, D. L. (1989): Genetics of angiosperm pollen. In: Scandalios, J. G. (ed.), *Advances in Genetics*. Academic Pres, Inc., CA. pp. 1–64.

Pepó, P., Szabó, E. (1998): RAPD markerek öröklődése kukoricában (*Zea mays* L.). (Inheritance of RAPD markers in maize (*Zea mays* L.).) IV. Növénynemesítési Tudományos Napok, MTA, *Abstracts*, 122.

Rice, T. B., Reid, R. K., Gordon, P. N. (1978): Morphogenesis in field crops. pp. 262–277. In: Hughes, K. W., Henke, R., Constantin, M. (eds.), *Propagation of Higher Plants through Tissue Culture*. National Technical Information Service, U. S. Department of Commerce, Springfield, VA, USA.

Smith, G. A. (1986): Sporophytic screening and gametophytic verification of phytotoxin tolerance in sugarbeet (*Beta vulgaris* L.). pp. 83–88. In: Mulcahy, D. L., Mulcahy, G. B., Ottaviano, E. (eds.), *Biotechnology and Ecology of Pollen*. Springer-Verlag, New York.

Springer, W. D., Green, E. C., Kohn, A. K. (1979): A histological examination of tissue culture initiation from immature embryos of maize. *Protoplasma*, **101**, 269–281.

Tanksley, S. D., Zamir, D., Rick, C. M. (1981): Evidence for extensive overlap of sporophytic and gametophytic gene expression in *Lycopersicum esculentum. Science,* **213**, 453–455.

Tomes, D. T., Smith, O. S. (1985): The effect of parental genotype on initiation of embryogenic callus from elite maize (*Zea mays* L.) germplasm. *Theor. Appl. Genet.,* **50,** 505–509.

Tóth, S., Bódi, Z. (2006): Indukált mutációval létrehozott kukoricagénbank a nemesítési alapanyagbázis növeléséért. (Gene bank developed by induced mutation for selection.) *Acta Agraria Debreceniensis*, **19**, 45–49.

Widstrom, N. W., Wilson, D. M., McMillian, W. W. (1984): Ear resistance of maize inbreds to field aflatoxin contamination. *Crop Sci.*, **24**, 1155–1157.

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TESTING OF MAIZE FOR REGISTRATION IN THE NATIONAL LIST IN GERMANY

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In Germany the Federal Office of Plant Varieties (Bundessortenamt) is responsible for variety testing and registration in the National List. The regulations of the German Seed Act follow the rules of Council Directive 2002/53/EC on the common catalogue of varieties of agricultural plant species. The German testing system is explained in detail.

Key words: national list, value for cultivation and use, variety registration, descriptive variety list

Introduction

Seed and plant regulations are very much harmonized throughout the European Union.

The regulations of the German Seed Act and those of all other EU member states follow the rules of Council Directive 2002/53/EC on the common catalogue of varieties of agricultural plant species and of Council Directive 2002/55/EC on the marketing of vegetable seed.

Both directives are for the benefit of seed consumers and are to ensure that agriculture and horticulture are supplied with high quality seed of well performing varieties. They therefore stipulate that – with certain exceptions – in all EU member states seed of agricultural species and vegetables shall not be offered for sale unless the variety concerned is registered in the National List of the member state in question, or unless, by way of registration in the National List of some other member state, it is contained in one of the Common Catalogues for agricultural species or for vegetables.

A variety has to be registered nationally first before it can be included in the Common Catalogue.

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If a variety is genetically modified it may only be registered if a "placing on the market" permission has been granted according to part C of Council Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms. In the case of a product for food or feed a permission according to Council Directive 1829/2003/EC on genetically modified food and feed is also a requirement for registration.

According to the above-mentioned directives all member states have to follow the same rules for the examination and registration of a variety.

A variety has to have a suitable denomination, it has to be distinct, uniform and stable and in the case of an agricultural species it has to have a value for cultivation and use in order to be registered in the National List. The basic principles for the testing of these requirements are included in the following directives:

In Council Directive 930/2000/EC the regulations for the suitability of a variety denomination for agricultural and vegetable species are stated.

Council Directives 2003/90/EC and 2003/91/EC give a detailed description of which guidelines have to be used for the examination of distinctness, uniformity and stability.

For testing the value for cultivation and use of agricultural species the council directives are not so precise. Here, according to Directive 2003/90/EC, the possible yield, resistance against damaging organisms, environmental behaviour and quality have to be assessed, but no clues to the guidelines, number of locations or duration of tests are given. Consequently, value testing is quite different in the member states.

As the definition of the value for cultivation and use given in Directive 2002/53/EC is also not very precise it leaves the member states to decide whether a quite open or more restrictive acceptance policy is applied in the question of agricultural value.

In the following the German testing system for the registration of maize varieties is explained.

Variety testing and registration in Germany

In Germany the Bundessortenamt (Federal Office of Plant Varieties) is responsible for variety registration in the National List according to the Seed Act. The Federal Office of Plant Varieties is an independent federal authority under the supervision of the Federal Ministry of Food, Agriculture and Consumer Protection.

An application for the registration of a variety in the National List can be lodged by any person or firm within the EU; others can lodge an application by means of a representative from the EU. All applications are published.

Maize is a very important species in German agriculture. The cultivation area in 2005 amounted to 1,260,000 ha for silage utilization and to 450,000 ha for grain utilization. Every year applications for registration in the National List are made for around 150 new maize varieties.

Testing of distinctness, uniformity and stability

The examination of the distinctness, uniformity and stability (DUS test) of varieties that have applied for registration starts at the same time as the testing of the value for cultivation and use (VCU) and usually needs 2 years. Not only the hybrid but also the genealogical components (inbred lines, single crosses) of a variety are tested. The DUS tests are conducted at two testing stations of the Federal Office of Plant Varieties. The testing protocol follows the guidelines established by the Community Plant Variety Office (CPVO).

Testing of the value for cultivation and use

The aim of the value tests is to find out the agricultural value of a variety, that is, the sum of its agronomic characters, susceptibility to diseases, and yield and quality parameters.

Depending on the information given in the application each maize variety is tested for silage and/or grain utilization. Testing is done in the maturity classes early (≤ 220), mid-early (230–250) or mid-late to late (≥ 260).

The normal duration of the VCU tests for maize is two years.

Because of the large number of varieties for testing, new candidates and varieties in the second year have to be tested in two different series. The first-year series is run at 14 trial sites, mainly at breeding stations. The second-year varieties are tested at 18 trial sites, mainly at institutions of the Federal States. The locations are distributed in the climatic zones typical for the maturity class in question.

Depending on the number of varieties the value test is designed and evaluated as a randomised block or in a lattice design (more than 40 varieties). In each trial series 6–7 standard varieties are sown. The trial is one-factorial with three replications per variety.

Sowing is done with a higher density than the desired number of plants. The final number of plants per plot is achieved by thinning.

The plot size at sowing must be at least 18 m^2, with four rows. Only the two inner rows (9 m^2) of the plot are observed and harvested in order to avoid border effects.

The following characters of cultivation, yield and quality are observed.

Chilling sensitivity (1–9), tillering ability (count, 1–9), silking date, plant height (cm), European corn borer (*Ostrinia nubilalis*) (count), maize smut (*Ustilago maydis*) (count), stalk rot (*Fusarium*) (count), *Helminthosporium turgicum* (1–9), tendency to lodge (count), number of plants before harvest, ripeness of leaves (only silage, 1–9). Other characters are registered when they occur.

For silage utilization it is time to harvest when the standard varieties have reached 30 to 35% dry matter in the whole plant. The harvested material is evaluated for the dry matter content (basis for judgement and the later description of the maturity of a variety) as well as for the dry matter yield of the whole plant. Samples are taken from the harvested material to be evaluated for digestibility (ELOS) and starch content using the NIRS method in the laboratory.

For grain utilization it is time to harvest when the standard varieties have reached 60% dry matter in the grain. The dry matter content of the harvested material is the basis for judgement and the later description of the maturity and is necessary to determine the grain yield. Samples are taken to be evaluated for the thousand grain mass and the share of broken grains in the harvested material.

If a variety does not fit in the maturity class it has been entered for, the variety either has to be withdrawn or has to be retested in the right series.

After each year the VCU test results are computed using electronic data processing equipment and are compiled in detailed value test reports. The yearly report is given to all breeders concerned over the Internet (password is necessary).

With the help of the report the breeder can compare the performance of his variety with the standard varieties as well as with the other candidates. In order to facilitate the breeder's decision an index calculation is done additionally. Poor varieties are normally withdrawn by the breeders.

Decision on registration

At the end of the two years of value testing a summarized report with all results is issued and the breeder is invited to discuss his variety with the variety committee.

The decision on variety registration is made by a variety committee which consists of three members of the Federal Office of Plant Varieties.

In order to be registered the variety has to have a denomination, it has to be distinct, uniform and stable and it has to have a value for cultivation and use. The German Seed Act states that a variety has a value for cultivation and use if – in comparison with similar varieties registered in the National List – the total of its characteristics clearly improve crop cultivation or the utilization of the harvested crop or any product obtained from such a crop. The improvement shall be given at least in a defined region. Inferior characteristics may be disregarded where other superior characteristics are present.

No index system is used for the decision.

If the variety meets all requirements it is registered in the National List for a period of 10 years. A prolongation is possible.

From the great number (around 150) of new applications for registration each year, between 15 and 30 maize varieties (10–20%) are registered. The rest of the varieties are withdrawn during the two-year testing period or are rejected by the variety committee.

In the case of a rejection, the breeder has the right to appeal. After the decision of the appeal committee (7 members) the applicant has the right to appeal to an administrative court.

After a variety has been registered in the National List it is communicated to the European Commission in order to be added to the Common Catalogue. As soon as it is published in the Common Catalogue, certified seed of the variety may be marketed throughout the Union.

Value tests for recommendations to farmers

In the German testing system the Federal Office of Plant Varieties is in charge of variety testing for registration, while the Federal States (Laender) are in charge of variety testing for advice and recommendation to the farmers.

After their registration in the National List all varieties enter the variety trials conducted by the Federal States (federal variety trials). In these trials the varieties are tested for another two to three years before a recommendation to farmers is issued. Besides nationally registered varieties, interesting varieties from the Common Catalogue are also tested in a special system.

Descriptive Variety List

At the end of each year the results of all German variety trials are transmitted to the Federal Office of Plant Varieties and form the basis for the Descriptive Variety List (Beschreibende Sortenliste) issued yearly. These descriptive variety lists give a description of all registered varieties as regards characters important for cultivation and use. A comparison between older and younger varieties is possible. The Descriptive Variety List does not give a recommendation. For recommendations the farmer must contact the institution of his Federal State, which will give him regional trial results and advice.

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HARMONIZATION OF VCU TESTING METHODS FOR MAIZE VARIETIES IN A EUROPEAN CONTEXT

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In the European Community, a new variety of an agricultural crop must be submitted for official trials for DUS (Distinctness, Uniformity, Stability) and VCU (Value for Cultivation and Use) before commercialization. The guidelines for these tests are summarized in the European directive 70/457/EU (1970), revised in 2002 (2002/53/EU).

At present each EU country has a separate system for VCU testing. The EU directive stipulates that the VCU value must be satisfactory. The term “satisfactory” can be interpreted in different ways, so the level for admission for the same varieties may differ greatly between countries. For the market this can lead to a different assortment of varieties, adapted for the same ecological regions, but distributed over several countries.

The different steps, from acceptance of a variety for trials, through the organization, the evaluation of parameters during the growing season, harvest modalities and data processing to the criteria for registration in Belgium are presented in the paper, followed by an analysis of the registration procedure in Belgium in comparison with other countries

Thereafter, a proposal is given for harmonization and international cooperation in the VCU testing of varieties adapted to comparable ecological regions of neighbouring countries and with the same crop exploitation and use of the final product. For these varieties it is important that nearly the same rules are used for the registration of VCU value.

The basis for successful international cooperation is a good knowledge of the national systems, searching for similarities and finding a solution for differences.

Key words: maize, variety testing, harmonization, EU directive

Introduction

In the European Community, a new variety of an agricultural crop must be submitted for official trials for DUS (Distinctness, Uniformity, Stability) and VCU (Value for Cultivation and Use) before the commercialization of seeds is possible. If both DUS and VCU are positive, the variety can be registered in a national catalogue in one EU country. National admission means that from that moment the variety can be multiplied (seed production) and commercialized. With the recently adapted European regulation, once a variety is admitted, it can be added to the European catalogue after a few months. This EU registration allows commercialization throughout the European community.

The guidelines for VCU and DUS tests are summarized in the European directive 70/457/EU (1970), revised in 2002 (2002/53/EU) (Anonymous, 2002). The aim of this directive is, on the one hand, to protect breeding companies (breeders' rights) and on the other hand to protect farmers by only registering varieties that are at least as good as existing ones. According to this EU Directive a large number of uniform criteria and minimum requirements must be laid down. Article 4 stipulates that the VCU value shall be regarded as "satisfactory" if, compared to other varieties accepted in the catalogue of the member state in question, its qualities taken as a whole offer, at least as far as production in any given region is concerned, a clear improvement either for cultivation or as regards the uses which can be made of the crops or the products derived therefrom. Furthermore, the procedure for the trials stipulates that current scientific and technological knowledge has to be taken into account.

Actual situation in different European countries

At present each EU country has a separate system for VCU testing. The EU directive stipulates that the VCU value must be satisfactory. The term "satisfactory" can be interpreted in different ways, so the level for admission for the same varieties may differ greatly between countries. Some countries interpret the directive very liberally (only considering risk parameters), thus making it possible to register any variety that offers possibilities for commercialization. Others go to the other extreme and have very strict criteria, so that the % of admissions will be rather low. The final aim is only to register varieties that could be recommended to farmers, based on a large number of data (several years of testing with many trials per year).

Based on these two philosophies the purpose of the VCU trials may be different (for example, choice of standard varieties, severity of criteria for registration, etc.). For the market, this can lead to a different assortment of varieties, adapted for the same ecological regions, but distributed over several countries.

Steps from acceptance for trials to the registration of new varieties in Belgium

This section presents the different steps, from acceptance of a variety for trials, through the organization, the evaluation of parameters during the growing season, harvest modalities and data processing to the criteria for registration in the Belgium system.

Acceptance of varieties for trials

Private or official breeding companies or their representatives may request the relevant authorities (Flemish and Walloon Regions in Belgium) to investigate their new candidate varieties of agricultural crops for DUS and VCU, according the EU Directive 70/457/EEC. The Minister (Flemish or Walloon) responsible for the edition of the national catalogue defines, at the suggestion of the Technical Interregional Working Group for Variety Testing (TIW, formerly Committee), the common and specific VCU tests and criteria that will be organized.

Based on the information provided by breeders the varieties are classified as "silage" or "grain". To enter for official trials there is no need to present agronomical data on the varieties when tested under local (national) conditions in previous years. The variety system also permits the testing of a variety for a specific characteristic or use (biological agriculture, energy maize, etc.). In such cases the protocol and trial conditions are elaborated by the TIW. For GMO varieties a specific procedure is set up.

Seed material and control of seed quality

From all varieties, new and standard, untreated seeds must be delivered by the breeders. The minimum requirements for germination capacity are based on a cold test, executed by the official institute (Van Waes, 1995). Only seed samples with an emergence higher than 85% of normal plantlets in the cold test are accepted for field tests. All seed lots delivered are treated by the official institute with the same fungicide.

Choice of standard varieties

At the beginning of a testing cycle (2 or 3 years) the standard varieties are fixed, in agreement with breeders and officials. The choice of the standard varieties is one important factor in determining the level of admission. The standard varieties do not change during the testing cycle. Each year there can be a change in standard varieties; this is mainly based on entering the best new registered varieties and removing the worst standard varieties of the last testing cycle. For both silage and grain maize, a new set of 8 standard varieties is used every year to compare new entries; the change is restricted to 2 or 3 varieties per year. These standard varieties are chosen on the basis of various criteria (earliness, yield, quality, resistance to diseases, harvest security, etc.).

Grouping of varieties in the trials and organization of the field trials

Separate testing systems are set up for silage and grain maize. Within each category all the new varieties are tested together. There is no split up into earliness groups in the field. The classification of the varieties as silage or grain, or both, is based on the information given by the breeders. There is in principle no restriction concerning the earliness of the varieties. The silage maize varieties presented have a range of dry matter content in the total plant of 25–37%. For grain maize the range is between 60 and 73% dry matter in the grains.

For each purpose, silage or grain, the varieties are grouped in the field per testing year. So in each location there are 3 trials for silage (1st, 2nd and 3rd year) and the same for grain maize. All plots have 4 rows; only the 2 central rows are taken into account.

Sowing density, plant density and thinning out

The sowing density is based on the germination percentage in the cold test (Van Waes, 1995). In general the sowing density is 15% higher than the assumed plant density. The purpose of the trials is to evaluate all varieties at the same density. To attain the assumed plant density thinning is carried out in the 3–4-leaf stage. For silage and grain maize the density is 100,000 and 90,000 plants per ha, respectively, but this also depends on the sowing date (for silage maize: sown before 10th May: thinning to 100,000 plants/ha; sown after 10th May: 90,000 plants/ha; for grain maize: sown before 10th May: 90,000 plants/ha; sown after 10th May: 80,000 plants/ha).

Characteristics evaluated during the growing season, just before and at harvest

During the growing season the following characteristics are evaluated: early vigour, flowering date, total plant height, height of ear attachment. In this paper detailed information is only given about early vigour; for the other characteristics the protocol is described in the variety testing handbook (Van Waes et al., 2003).

Early vigour is estimated in the 5–6-leaf stage on a 1–9 scale (9 for the strongest development, 5 for moderate and 1 for the weakest development) derived from the UPOV directive for maize. This is based on the estimation of both lines and hybrids. In general lines have poorer early vigour than the slowest developing hybrids. Therefore, a score of 5 is given to the hybrids with the worst development.

Just before harvest the percentage of stalk rot and *Ustilago maydis* is counted per plot (replication). These figures are then used to calculate the percentage for the whole trial.

Observations on lodging and stalk rot are made not earlier than 3 days before harvest for silage maize. Maize plants are considered as lodged if they infringe on neighbouring rows, or if they are prostrate or broken. For grain maize lodging (mechanical) is evaluated two weeks before harvest, while stalk rot is scored just before harvest.

All the trials in the different locations where lodging or stalk rot is observed are taken into account if at least one variety shows more than 5% of lodged plants or plants affected by stalk rot (average of the 3 replications). Locations where the average lodging or attack by stalk rot of all varieties is higher than 50% are not taken into account.

Harvest modalities

Silage maize

The harvest starts from the moment that the reference standard variety in the mid-early group has reached the dough stage (28–30% dry matter content in the total plant), as proposed by TIW. At harvest the fresh weight of the total plant is noted per plot. A representative sample of 1.5 to 2 kg fresh material is taken to determine the percentage of dry matter in the total plant; this sample is also used for quality analyses.

Grain maize

The harvest starts from the moment that the reference variety in the mid-late group has reached a maximum of 35% moisture in the grains, as proposed by TIW. At harvest the fresh weight of the grains is noted per plot. A representative sample of 1.5 to 2 kg fresh material is taken to determine the percentage of moisture in the grains.

Validity of the trials

The validity of the trials is based on regularity in the field, on the harvest date and the related dry matter content and on a statistical analysis of the parameters for yield and for dry matter.

a.) Regularity during the growing season

Regularity in the field is mainly based on the plant density. On plots with very poor emergence (less than 90%), no further observations are made. The breeder or his representative is warned about this. In addition, growing conditions, for example drought during the growing season, may have a negative influence on regularity, possibly leading to a trial being rejected.

b.) Harvesting date

If the harvest has to be delayed due to bad weather conditions, a trial may be cancelled. The decision for silage maize is taken on the basis of the average dry matter content in the total plant for all varieties: if this is > 36%, then the trial is cancelled.

For grain maize varieties the trial results may be rejected if the average dry matter content of the grains of all varieties is ≤ 60%.

As an additional condition, trials where more than 50% of the plants show lodging or stalk rot, averaged over all the varieties, are not taken into account.

c.) Statistical analyses of the yield (total plant or grain) and % dry matter

The statistical analysis of the yield parameters is done using SAS software. The estimation is based on variance analyses. The variation coefficient (V.C.) is a norm for the regularity of the trial or the sampling technique. The variance analysis is done for the total dry matter yield for silage and on the grain yield for grain maize varieties. If the V.C. is > 8%, the trial is rejected. The variance analysis is also done for the dry matter content. If the V.C. is > 5%, the trial is rejected. It has to be stressed that both norms must be fulfilled (V.C. for yield and dry matter content) in order to keep or reject a trial.

Evaluation system and conditions for admission

An index system, based on the most important quantitative and qualitative characteristics, is used. The coefficients for the parameters in the index are based on correlations. New varieties are compared to eight standards, of which the best four, based on all their characteristics, are chosen for the final judgement. This index system is presented for silage and grain maize, respectively, in Tables 1 and 2. For harvest security parameters (resistance to lodging and stalk rot) new varieties are compared to the average of all standard varieties. The formula used is based on the acceptable level of lodging and/or stalk rot in practice before real losses can be established.

For the criteria for evaluation it is important that there is a good equilibrium between parameters for yield, harvest security (resistance to lodging and stalk rot), disease resistance and quality. At the beginning of the testing period the criteria and the potential standard varieties are fixed. They are not changed during the testing period. However, each year new varieties are introduced into the trials, at which time the standard varieties may change. These are in general the best registered varieties. So the level for judging new varieties will be higher.

The selection procedure for new varieties for listing in the national catalogue is stepwise. Half the varieties are rejected after the first year (too low agronomical value). After two or three years a new variety must be better than the average of the four best standard varieties for the total of all agronomical characteristics in the index before the variety can be registered.

Table 1

Index system for silage maize

Characteristic	Rating scale	Coefficient
Yield of total dry matter (kg/ha)	%*	+ 1.0
Earliness (% dry matter of total plant)	% (real figure)	+1.5
Susceptibility to lodging	% lodged plants	< 5 + 0.3 x**
Digestibility (% dry matter)	% (real figure)	+1.5

* relative figure in comparison with the 4 standards; **x = average % of standard

Table 2

Index system for grain maize

Characteristic	Rating scale	Coefficient
Yield of grains (kg/ha)	%*	+ 1.0
Earliness (% moisture in the grains)	% (real figure)	– 3.0
Susceptibility to lodging	% lodged plants	< 5 + 0.3 x**
Susceptibility to stalk rot	% plants with stalk rot	< 5 + 0.3 x**

* relative figure in comparison with the 4 standards; **x = average % of standard

Analysis of the different steps in the Belgian registration procedure and comparison with other systems

A non-restricted number of new varieties for the VCU trials could lead to too many varieties for a good practical organization of the trials. In this scenario there is also the possibility that some of the new varieties are not really potential improvements for agriculture. The reasons for this may differ: not adapted to the specific conditions (too late ripening) or to the local agricultural practice; too low agronomical value compared to the standard varieties. To avoid this either the price per variety for VCU testing can be raised and/or the results from previous testing could be required. On the other hand, this scenario of a non-restricted number of varieties would help to attract varieties from a larger number of breeding companies, which could lead to broader genetic diversity in the trials. More genetic diversity could be of interest for agricultural practice in the case of risks for specific diseases.

The aim of variety testing should be to test the genetic differences between varieties and not the effect of different treatments. It is known in general that seed treatment may differ considerably between companies and that the effect on early vigour, for example, may be important, thus giving some varieties an advantage, independent of their genetic composition. Starting from this point of view it is logical to have a delivery of non-treated seeds (for both new and standard varieties) for the official tests, but as only treated maize seeds are used in practice to protect them against soil fungi and bird damage in the emergence stage, it is also obligatory to use treated seeds for the VCU trials, but to do this by a uniform method at the official institute.

In the Belgian system the breeder is informed if the cold test gives less than 85% emergence and he is requested to send a new seed lot. If the new result is not better, the candidate variety will not be tested. The reason is that poor emergence in the field will influence not only the results of the variety itself, but also those of surrounding varieties.

In order to exclude the year influence (e.g. weather conditions), variety trials are organized over at least three different growing seasons. This means that variety trials with the same candidate varieties and the chosen standard varieties are organized in three consecutive years at different locations, representing the most important soil types for agricultural purposes in the country. During a testing cycle of three years there is a good chance that clear differences between the varieties will be detected even for harvest security parameters (lodging, stalk rot).

The choice of standard varieties has an important impact on the level for judging the new varieties. At least two different scenarios are used in the EU countries. In the first scenario the standard varieties are chosen from among the best new and old varieties in the catalogue. A quick change in standard varieties, by continually using the best new registered varieties, puts the admission level every year somewhat higher. This can result in a low percentage of new registrations. One advantage of this system is that farmers can continuously profit from the progress in breeding, because only the best varieties are registered, which can be directly recommended. One disadvantage could be a very rapid change of varieties on the market, before the farmers can really decide whether the varieties suit their specific conditions. In addition, it is clear in some cases that the new standard varieties have very high potential but that they are not always very stable under stress conditions. Furthermore, it is important for standard varieties to be a reflection of what is actually cultivated or will be cultivated in the future. So it is important that the choice of new standard varieties should be based on at least the following parameters: minimum three years of testing, agronomical stability, choice made in consultation with breeding companies, so that only varieties cultivated in practice will be chosen and not “theoretically high yielding” varieties that are not of interest for seed production.

In the second scenario widely cultivated, stable varieties are used as standards. These varieties are in general somewhat older and in most cases have a lower yield compared to recently registered varieties. For maize new varieties only stay at the top for 3 to 4 years because the progress in breeding is extremely rapid (Van Waes et al., 1994; 2005). As a consequence of using older varieties as standard the level for comparison will be not so high for new applications. So, depending on the norms for registration, more or fewer varieties will be added to the catalogue, thus allowing them to be commercialized. If the same registration norm is used as in the first scenario, more varieties will be registered, in which case a large number of new varieties will be introduced into the market, only some of which represent real improvements and could be recommended

Current maize varieties have in general very good resistance to lodging and stalk rot. These characteristics cannot be judged in a single growing year. Therefore, observations over at least three years are obligatory for the judgement of stalk rot and lodging, which are criteria for the rejection of a variety. If stalk rot and lodging cannot be recorded because of their absence, neither characteristic is taken into account in the final judgement.

The use of an index system, as in Belgium, with fixed criteria and standard varieties at the beginning of the testing cycle, offers a reference point for breeders. They know the rules for registration before they enter a new variety in the trials and they can calculate the chances of registration. It also provides a reference point for TIW. Normally varieties which score lower than the standard will not be proposed for registration; so an index system is not very flexible if a variety scores just below the index. Furthermore, it not always easy to fix the coefficient between the parameters in the index. However, the Belgian system also offers the possibility to register a variety for one specific characteristic, even if the other characteristics are somewhat below the norm.

In the recently revised criteria a distinction is made between quantitative and harvest security parameters. The criteria for registration may push breeding companies in a certain direction. Variety research stands between agricultural practice and breeding, as shown in Figure 1. The evaluation criteria are based on the most important characteristics for agricultural practice. On the basis of the criteria for the release of new varieties, variety research can contribute to sustainable, biological agriculture and/or anticipate new situations. New quality criteria for the introduction of new varieties of maize could be: lower input of fertilisers and herbicides (better early vigour), biological agriculture, cold tolerance in spring, quality characteristics and disease resistance. Before the incorporation of new criteria their impact on variety release has to be studied.

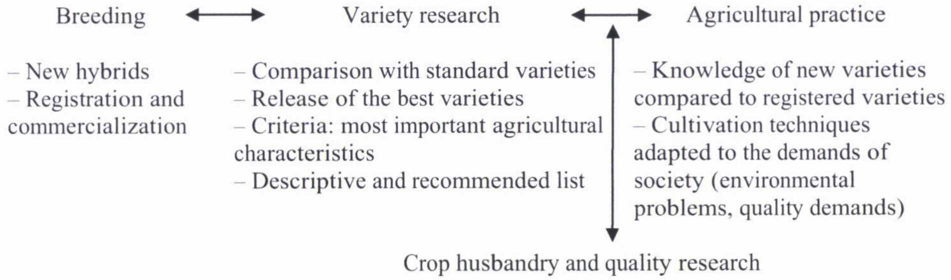

Fig. 1. Interaction between variety research, breeding and agricultural practice

A norm for refusal after one year results in keeping only the best new varieties in the trials (potential improvements). The possibility of registering varieties after only two years of VCU trials if the final result in the index is above the norm, can lead to the rapid introduction of new, potentially very good varieties into practice. This is favourable for both farmers and seed companies.

Another difference between EU countries is the number of standard varieties. A large number of standard varieties means higher costs for testing. On the other hand, the use of more standard varieties, from which the average value of different agronomical parameters is used to compare new varieties, results is a more stable basis for judging. In addition, from the statistical point of view, more standard varieties can have the same effect as a supplementary repetition.

To offer the best varieties to the market, the aim of VCU research should be to predict the agronomical and technological value of a new variety in a reliable and neutral way in comparison with standard varieties. Therefore, a large number of trials and quality analyses per year are necessary. In the case of silage and grain maize, 7 and 6 trials, respectively, were set up in the different agricultural regions of Belgium. This implies high national experimentation costs.

The costs of a well-organized variety testing system are, however, very low compared with the benefits. For silage maize the costs are only 6% of the benefits. For grain maize they are somewhat higher (10%), but still only a fraction of the potential benefits.

Proposal for harmonization and international cooperation

A proposal for harmonization and international cooperation for varieties adapted to the comparable ecological regions of neighbouring countries and with the same crop exploitation and end use will be elaborated in this section.

The basis for successful international cooperation is a good knowledge of the national systems: searching for similarities and finding a solution for differences.

The most important similarities between the different national variety testing systems are: evaluation of new varieties in comparison with standard varieties, comparable field planting protocols and harvest modalities, and judging of much the same characteristics: yield, earliness, resistance to lodging and stalk rot.

Important differences are: a different system for the registration of varieties in the national catalogue (index of characteristics, level of standard varieties), a different method of seed preparation (disinfection), evaluation for earliness: all groups together or separately, plant density as a function of the earliness group, different quality characteristics and methods of analysis (classical wet analyses, NIRS).

The aims of international cooperation for VCU tests should be: i.) to predict the agronomical value of a new variety for the whole agro-ecological zone where the variety can be cultivated, and ii.) to predict the VCU value with the same reliability as at the national level. This second point is very important for the users (farmers) of the new varieties to have objective data about the potential of new varieties.

To harmonize variety testing at the EU level the following steps are proposed:

Step 1: Definition of agro-ecological zones

Step 2: Evaluation of the national systems (preparation of seeds and field protocol, evaluation criteria) for countries with comparable ecological regions

Step 3: Comparison of data of common varieties for different quantitative and qualitative characteristics

Step 4: Data processing based on the national systems and on the whole agro-ecological zone

Step 5: Selection of locations for an international network (genotype × environment interactions)

Step 6: Planting of an international network with common varieties

Step 7: Evaluation of the results of this network after a testing cycle of 2 to 3 years

Step 8: Proposal for adaptation of the EU rules for direct registration in a European catalogue.

The first step, namely to define the agro-ecological zones, is extremely important. Three criteria should be considered: a) Which maize type can be cultivated (in terms of earliness)? b) What is (are) the current practice(s) in the potential cultivation zone? c) Which criteria are important for variety release in the defined agro-ecological zone? The whole zone may be theoretically good for the cultivation of a variety with a specific FAO index, but other climatic conditions (differences in disease pressure, high risk of storms in autumn) may lead to different criteria for the registration of varieties and to a split-up of the agro-ecological zone. As a model for defining agro-ecological zones heat units (Fig. 2) or the FAO index for earliness (Fig. 3) can be used. In the second model, there is considerable overlapping between the zones.

The outcome of an international variety testing network will be a broader basis for the national authorities for judging the VCU value of a new variety, achieved with lower national testing costs. For registration two options are still possible: i.) national registration based on the data of a common network, still leaving the possibility to accentuate important agricultural parameters in a different way per country; ii.) direct registration at EU level, based on a common protocol and criteria.

The results of testing varieties in agro-ecological zones, over national borders, will be a win-win situation for all partners in the chain of variety testing.

For the breeders the registration costs will be lower, due to the fact that only one application will be necessary for the European catalogue, while at present a breeder has to pay per country when he wants to test a variety at the same time in several EU countries. Furthermore, the breeders will have data for the whole zone in which the variety can be cultivated; this offers more possibilities for the commercialization of seeds.

For the national institutes responsible for organizing official trials, international cooperation could result in lower experimentation costs (reduced number of trials per country), while maintaining the same level of reliability for the prediction of the performance of a new variety.

Based on an international network it will be possible to provide farmers with neutral, objective information on all the varieties that can be cultivated in a given agro-ecological zone. There will not be different data for the same variety in the same agricultural region over several countries.

Conclusions

Variety research is regulated at the European level and is important for both breeders and farmers. Until now the VCU trials have been organized at national level and not in relation to agro-ecological zones. The differences in the registration procedures of EU countries may lead to a different assortment of varieties on the market, even when they were tested in the same ecological regions.

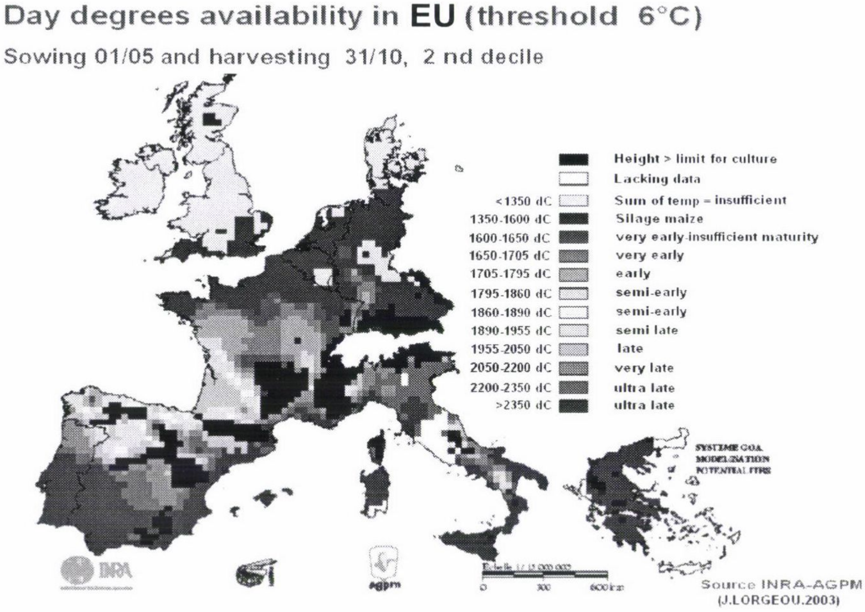

Fig. 2. International cooperation – Agro-ecological zones (Source: INRA – AGPM)

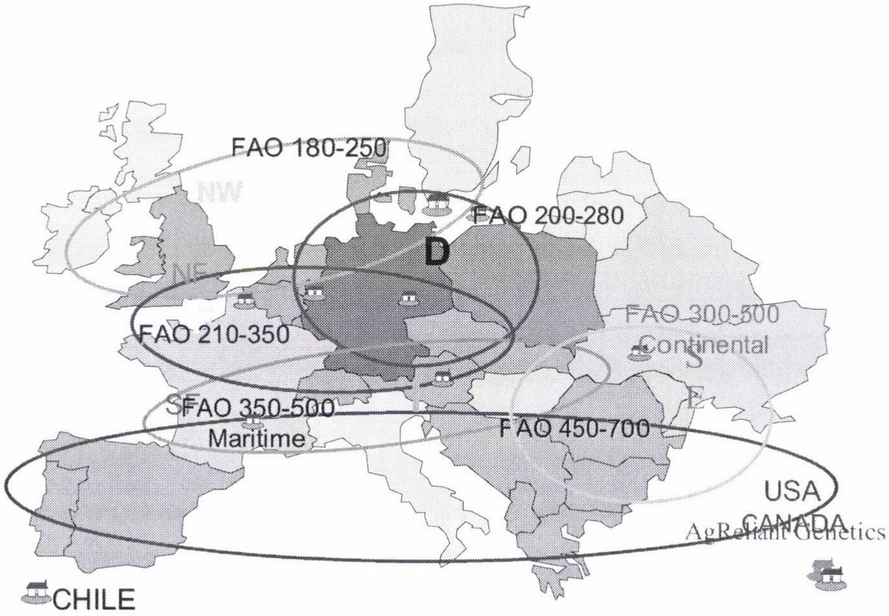

Fig. 3. International cooperation Agro-ecological zones – Basis FAO (Source: KWS)

International cooperation for field trials, based on agro-ecological zones, offers a way to reduce experimentation costs. Before an international network can be operational, various steps will have to be taken, leading finally to direct registration in the European catalogue. This will be a win-win situation for all partners dealing with variety research (breeders, official institutes, farmers, agro-industry).

New varieties only stay at the top for 3 to 4 years. For agricultural practice a continuous switch-over to the best new ones is necessary to profit from the progress in breeding. A good variety choice is an important factor in the cost-effectiveness of maize growing.

References

Anonymous (2002): Council directive 2002/53/EC of 13th June 2002 on the common catalogue of varieties of agricultural plant species. *Official Journal of the European Communities*, **L 193**, 1–11.

Van Waes, J. (1995): The use of a cold-test to predict the field emergence with maize in official tests in Belgium. *Seed Science and Technology*, **23**, 211–224.

Van Waes, J., Carlier, L., Ilersic, J. (2003): *Handbook for Good Field Practices for Variety Testing and Post Control Trials of Agricultural Crops*. DFE-CLO, Ghent, 59 p.

Van Waes, J., De Bel, N., De Vliegher, A., Carlier, L., Herman, J. L. (2005) : *Catalogue belge des variétés de plantes fourragères et engrais verts: description et recommendation*. 5th edition. DFE-CLO, Ghent, 103 p.

Van Waes, J., Van Bockstaele, E., De Vliegher, A. (1994): Evolution of quantitative and qualitative characteristics of forage maize during the last 20 years in Belgium. *Acta Horticulturae*, **355**, 109–116.

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EVALUATION OF SOUTH AFRICAN SORGHUM LANDRACES AND BREEDING OF VARIETIES SUITABLE FOR LOW-INPUT AGRICULTURE

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A large number of sorghum landraces possessing superior grain quality but poor yield potential are cultivated in South Africa, where sorghum is of regional importance as a main staple food. Agronomic traits of landraces and newly developed breeding lines from Southern Africa were evaluated under low-input and optimal conditions. Molecular evaluation was carried out on the basis of AFLPs and SSRs. The accessions clustered into two groups. Mean genetic similarity was estimated at 0.85 using AFLPs and 0.31 using SSRs. Genetic diversity was calculated at H=0.136 and DI=0.597 for landraces and H=0.140 and DI=0.580 for breeding varieties. The most promising accessions concerning yielding ability and grain quality were selected and introduced to a breeding programme.

Keywords: *Sorghum bicolor,* genetic diversity, low input agriculture

Introduction

Sorghum is an important staple food throughout semi-arid Asian and African regions. Landraces, well adapted to low-input conditions as well as to biotic and abiotic stress factors and exhibiting superior grain quality, are still cultivated. The low productivity of grown varieties, frequent occurrences of food shortage in sorghum-growing areas, and the extension of sorghum cultivation to marginal lands, requires extensive breeding programmes followed by the introduction of new varieties fitting small-scale farmers' needs (Haussmann et al., 2000).

Knowledge of genetic diversity has an important impact on the improvement of crop productivity as well as on genetic resources conservation, in addition to the evaluation of agronomic traits (Simioniuc et al., 2002). Landraces may bear advantageous genes, useful especially in resistance breeding and in terms of quality traits (Tanksley and McCouch, 1997). Knowledge of

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genetic variation and the genetic relationship between genotypes is an important prerequisite for the efficiency of plant breeding programmes (Russel et al., 1997). In the frame of a breeding programme detailed knowledge on the genetic diversity within genepools facilitates a more efficient selection of parental genotypes. Loss of sorghum landraces and genetic resources is evident due to the replacement of landraces by new cultivars (Jordan et al., 2003). The potential of local sorghum landraces in developing cultivars with improved agronomic and food processing properties was demonstrated by Toure et al. (1998). Molecular markers are powerful tools for the assessment of genetic relatedness. AFLPs are advantageous due to the high number of polymorphic bands per assay unit (Bohn et al., 1999), whilst SSRs are highly polymorphic. Based on genetic relationships and yielding ability, hybrid performance was analysed by Ajmone Marsan et al. (1998) and the potential of sorghum hybrids in rain-fed agriculture in semi-arid areas was investigated by Haussmann et al. (2000). However, most new cultivars introduced in Sub-Saharan Africa are open-pollinated (Ahmed et al., 2000) and most small-scale farmers are not able to afford hybrid seed.

The main objectives of this study are therefore (1) the agronomical and genetic evaluation of sorghum landraces and modern cultivars grown in Southern Africa in order to detect the most promising accessions suitable for cultivar improvement, i.e. combining high yielding ability under low-input conditions with superior grain quality, (2) the establishment of advanced backcross systems using molecular tools for the identification of the genetic portion of the recurrent parent in the BC_1.

Materials and methods

Agronomic evaluation

The 46 sorghum accessions consist of 23 landraces from Southern Africa, from which 11 landraces were collected in the Northern Province, South Africa. Cultivated sorghum landraces in Southern Africa are represented by 12 additional accessions. Thirteen cultivars and breeding lines were obtained from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT). Five newly bred varieties each from the Grain Crops Institute, Potchefstroom, South Africa and from the Department of Crop Science, Maseru, Lesotho were included. Field trials including actual grown landraces and modern cultivars were carried out at two locations and planted in a randomized block design with three replications under low-input conditions, following the common practices of South African small-scale farmers (Pietersburg trials) and cultivation practices including use of chemical fertilizers, pest management and irrigation (Potchefstroom trials, for detailed information refer to Wenzel et al., 2001).

Molecular evaluation

Comparative molecular analysis using 28 informative AFLP primer combinations and 25 polymorphic SSR primer pairs were carried out in order to estimate the genetic relatedness. DNA isolation as well as AFLP and SSR analyses was carried out as described by Uptmoor et al. (2003). Genetic similarity was estimated according to Nei and Li (1979). Genetic diversity (H) on the basis of AFLP data was computed according to Lynch and Milligan (1994). The mean diversity index (DI) based on SSR data was estimated following Nei (1973).

The most promising cultivars regarding yielding ability and grain quality were chosen and introduced into a breeding programme. In order to reduce the number of backcross cycles, the genetic portion of the recurrent parent G(RP) was determined in BC_1. For this purpose, 92 BC_1 plants of two crosses were tested on the basis of 10 SSR and 16 AFLP primers. Field trials were carried out in Potchefstroom, South Africa under low-input rainfed conditions in a randomized block design comprising three replications using BC_1S_1 single plant progenies. Neither pesticides nor fertilizers were applied.

Results

Field trials

Yields in Potchefstroom were significantly higher than those in Pietersburg. In Potchefstroom the highest grain yields were observed for accession IS4394 (4.03 t ha^{-1}). Average grain yield was 2.97 t ha^{-1}. Grain yield in Pietersburg ranged from 0.44 t ha^{-1} to 2.85 t ha^{-1}; average yield was 1.21 t ha^{-1} (Fig. 1). Drought-resistant varieties produced higher yields under moisture stress due to increased tillering and reduced losses in seed number and seed mass. Thousand-seed mass (TSM) in Pietersburg was on average 19.3 g, ranging from 10.7 g to 32.0 g. Average TSM in Potchefstroom was 23.9 g, ranging from 13.4 g to 35.4 g (Fig. 1). Differences in TSM between the rain-fed and irrigated trials were statistically not significant. Continued precipitation during grain filling resulted in the softening of the kernel endosperm. Only IS4394, known for its hard endosperm, exhibited above average hardness. High humidity resulted in the leaching of pigments from the glume to the pericarp and endosperm, and in increased fungal growth. On average, brown (high tannin) and red kernels exhibited least kernel discoloration.

Genetic relatedness

The total number of scored bands on the set of 46 genotypes was 1135 based on 28 AFLP primer combinations; 61.76% of the AFLP bands were polymorphic. On 25 SSR loci 217 alleles were detected. The average number of scored bands was 39.3 for each AFLP primer combination. The mean number of alleles per SSR locus was 8.68, ranging from 3 to 20. Based on these data, total genetic diversity was estimated at H=0.167 on AFLPs and DI=0.665 on SSRs (Table 1). Within the subset of landraces and breeding lines genetic diversity was calculated at a similar level, i.e. H=0.136 (AFLPs), DI=0.597 (SSRs) for landraces and H=0.140 (AFLPs), DI=0.580 (SSRs) for breeding varieties (Table 1). Within the subset of landraces less genetic diversity was found in those derived from the Northern Province, i.e. H=0.073 (AFLPs) and DI=0.451 (SSRs). All sorghum accessions were uniquely fingerprinted by both marker systems. Mean genetic similarity was estimated at 0.85 using AFLPs and 0.31 based on SSRs. Spearman's rank correlation coefficient between genetic similarities assessed by SSRs and AFLPs was R=0.57. Principal coordinate analysis (PCA) revealed two main groups on both marker systems (Fig. 2). The strongest group separation was observed using AFLP markers. Most landraces are separated from cultivars and breeding lines. However, some landraces are closely related to the breeding varieties, while others (e.g. ZA0498) are genetically distinct from both groups.

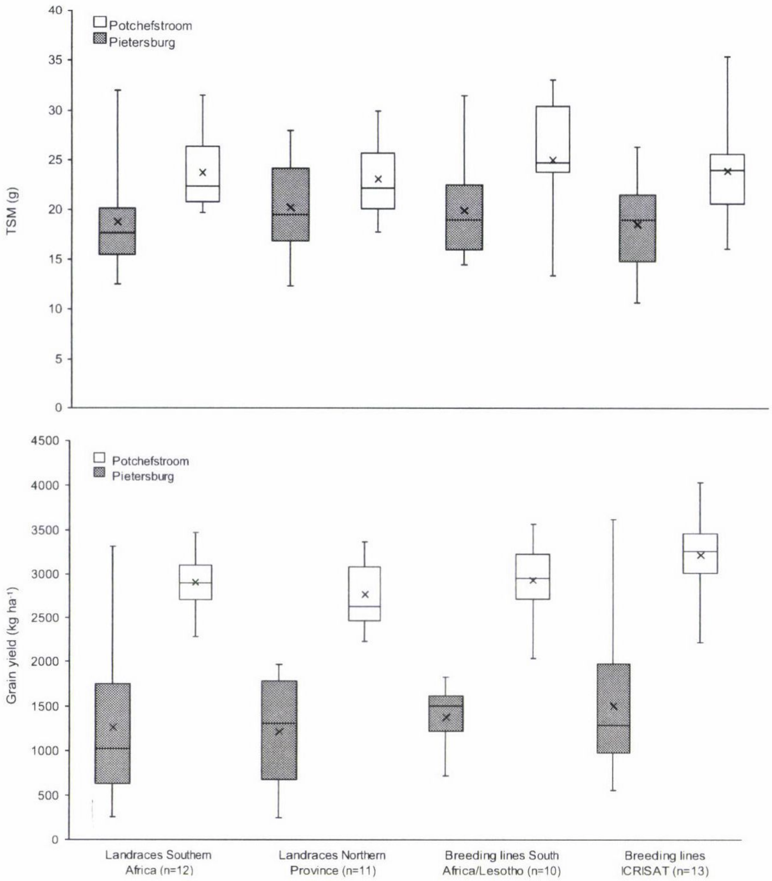

Fig. 1. Box and whisker plots for the variables TSM (top) and grain yield (bottom). Sorghum accessions are divided into four groups. Boxes represent Q_{75}, median, Q_{25}; whiskers represent max and min; means are given by x.

Table 1
Genetic diversity among sorghum accessions from Southern Africa

Sorghum accessions	N[a]	AFLP	SSR	
		H[b]	DI[c]	Alleles/locus
Landraces	23	0.136	0.597	6.56
Southern Africa	12	0.169	0.628	5.36
Northern Province	11	0.073	0.451	3.72
Breeding varieties	23	0.140	0.580	6.12
South Africa/Lesotho	10	0.141	0.517	4.20
ICRISAT/SMIP	13	0.136	0.563	4.61
All accessions	46	0.167	0.665	8.68

[a] N = number of accessions within each group or subgroup; [b] H = genetic diversity according to Lynch and Milligan (1994); [c] DI = genetic diversity (Diversity Index) according to Nei (1973)

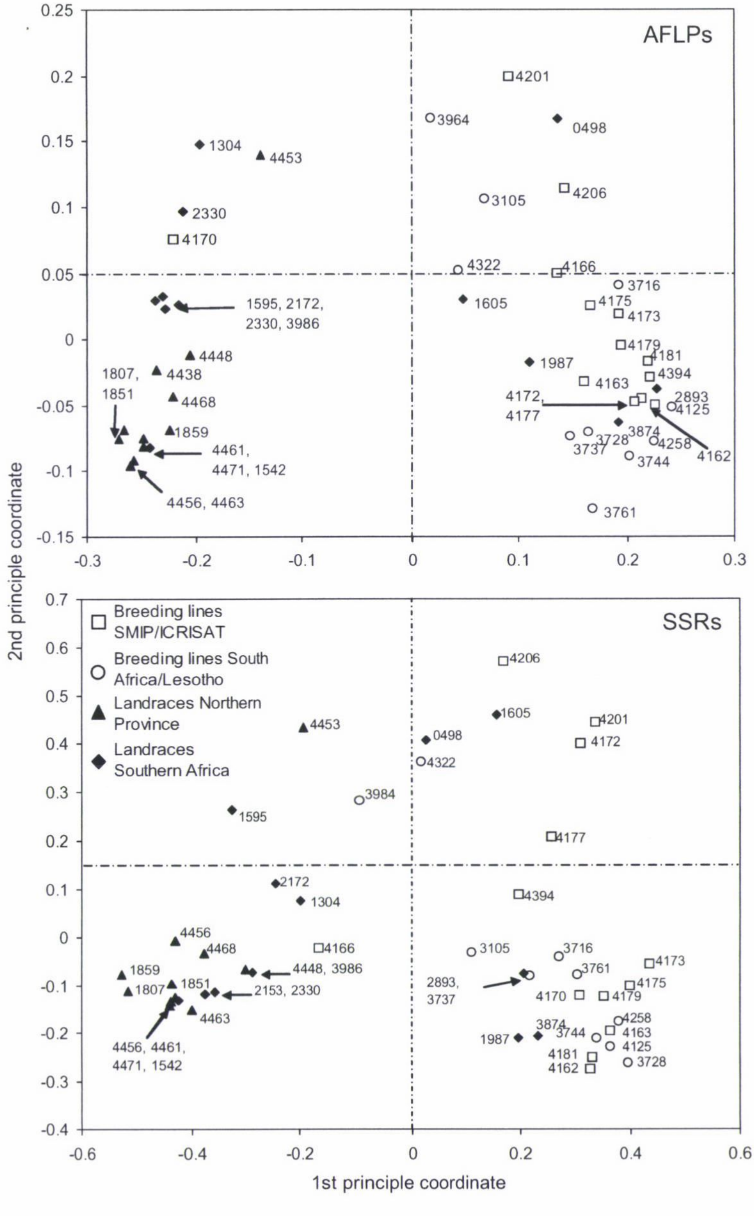

Fig. 2. Principal coordinate analysis on the basis of 28 AFLP (top) and 25 SSR markers (bottom)

G(RP) averaged 0.76 for (NP4453 × IS4394) × IS4394 and 0.74 for (LS3728 × IS4163) × IS4163 (Fig. 3). G(RP) varied from 0.53 to 0.95 in (NP4453 × IS4394) × IS4394 and from 0.60 to 0.86 in (LS3728 × IS 4163) × IS 4163. G(RP) was higher than 0.8 in 33% of the samples. In the cross (LS3728 × IS4163) × IS 4163 G(RP) was higher than 0.8 in 29% of BC_1 plants. G(RP) of

(NP4453 × IS4394) × IS4394 and (LS3728 × IS4163) × IS 4163, respectively, ranged between 0.7 and 0.8 in 43% and 49% of all plants. An average yield of 1.2 t ha^{-1} was observed in the (NP4453 × IS4394) × IS4394 families. G(RP) of the BC_1S_1 family revealing the highest yielding ability (2.2 t ha^{-1}) was estimated at 0.81. Grain yields of 52 (LS3728 × IS4163) × IS4163 families varied from 0.3 t ha^{-1} to 1.8 t ha^{-1}. G(RP) of the highest yielding BC_1S_1 family was estimated at 0.66. Yields of (LS3728 × IS4163) × IS4163 families averaged 1.1 t ha^{-1}. Pearson's correlation coefficient between yielding ability and G(RP) in BC_1 was negative (R=–0.30) for (LS3728 × IS4163) × IS 4163. Yield was found to be positively correlated with G(RP) in (NP4453 × IS4394) × IS4394 (R=0.44).

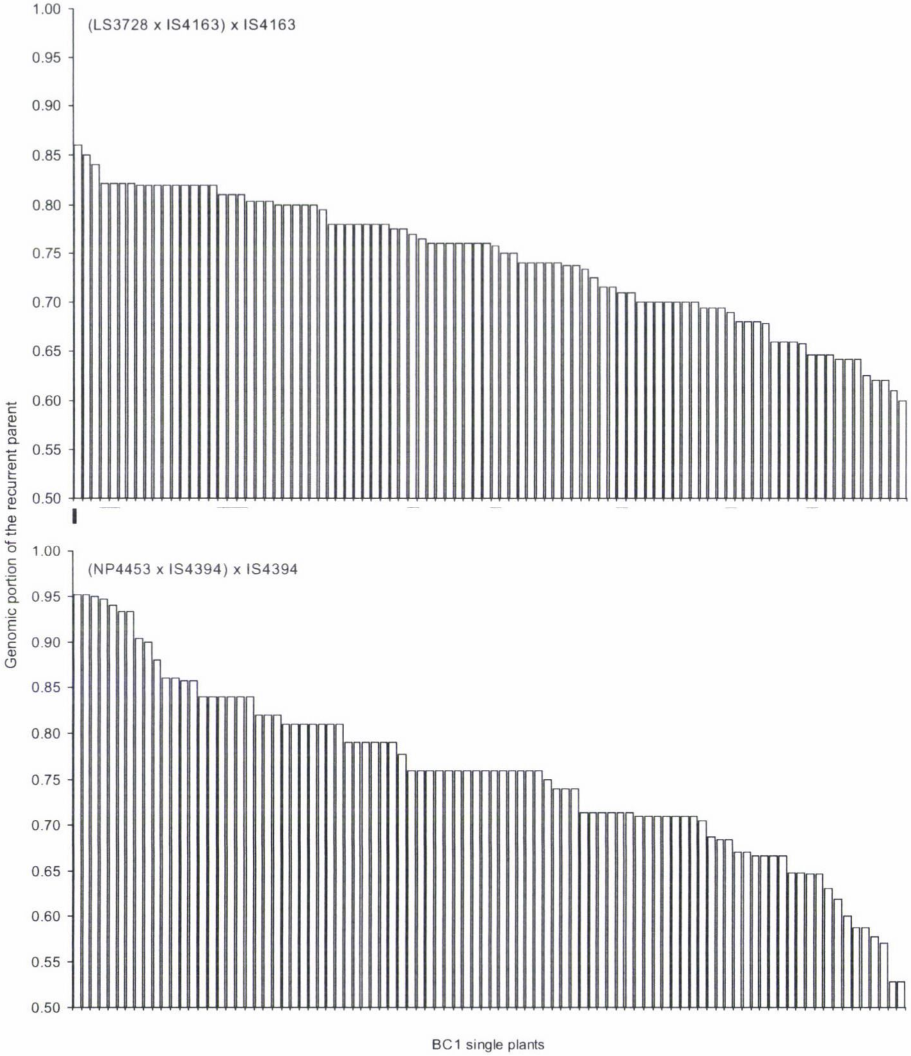

Fig. 3. Genetic portions of the recurrent parent in the BC_1 of the cross (LS3728 × IS4163) × IS4163 (top) and (NP4453 × IS4394) × IS4394 (bottom)

Discussion

Development in sorghum and pearl millet production in Sub-Saharan Africa follows two paths: intensive sorghum production utilizes hybrids, inorganic fertilizers, chemical pest management, and irrigation or water harvesting technologies (Ahmed et al., 2000). Hybrid seed production and successful marketing requires an effective seed industry, a good infrastructure, and a sufficient farmers' income to afford hybrid seed (Haussmann et al., 2000). Results indicate that improvement of cultivation practices can improve yields of cultivated landraces dramatically. However, in traditional South African small-scale farming systems, the lack of money is the major reason for reducing the application of inorganic fertilizers and sprayings to a minimum. The application of organic manure is limited by its availability and it is often used as a replacement for scarce firewood. Yield stability in semi-arid and arid regions is strongly correlated with drought resistance; therefore, cultivars with specific adaptation to extreme stress conditions and broad adaptation to climate differences between years are needed (Haussmann et al., 2000). Landraces, cultivated in drought-prone areas for generations, resulted from selection pressure under semi-arid conditions (Ceccarelli, 1994). In the present study, all landraces selected for drought resistance were late in maturity. Early maturing plants are better suited for drought escape in regions with short, irregular rain seasons. It is a common practice in semi-arid regions to cultivate both, early-maturing plants as part of a portfolio and late-maturing cultivars to take advantage of years with adequate rainfall (Ahmed et al., 2000). Prolonged drought during the pre-flowering period and excessive rain during post-flowering is common in the project area. It is assumed that pre-flowering drought resulted in reduced seed numbers, which was compensated by seed size due to excessive precipitation during grain filling. Wet conditions during ripening led to endosperm discoloration affecting flour quality in many accessions. The most important sensory attributes for porridge sorghum acceptance in West Africa were texture followed by taste and aroma. Appearance and colour were the least important attributes (Aboubacar et al., 1999).

With all the marker techniques the genetic diversity of landraces and breeding varieties was estimated at the same level, except for the group of landraces derived from the Northern Province. Low diversity within that group could be a result of the restricted geographic area. However, Djè et al. (1999) estimated a much higher diversity within 25 sorghum landraces derived from a restricted area of North Western Morocco. Genetic diversity was at a similar level as that estimated on breeding lines and landraces from Sudan (Abu Assar et al., 2005), while the genetic diversity estimated on all accessions was low compared to the results of Djè et al. (2000) and Grenier et al. (2000). These studies used sorghum accessions derived from different parts of the world and more informative SSRs; e.g. the five SSRs analysed by Djè et al. (2000) revealed on average 19.2 alleles on 25 accessions, while in the present study on average 8.68 alleles per SSR locus were detected. The correlation coefficient between SSR- and AFLP-based genetic similarities was low. Russell et al. (1997) found high correlation coefficients when comparing SSR with AFLP data, whilst Bohn et al. (1999) reported low correlations and speculated that this may be due to a low linkage between marker loci from different marker systems, resulting in the sampling of different genome parts. Goldstein et al. (1995) found that the average squared distance of microsatellites had, in contrast to simple allele sharing, an almost linear relation to time. This method was superior especially when applied on distantly related genotypes. The present studies revealed a stronger correlation using the squared average as a distance measure between SSRs and AFLPs (data not shown). Groups of landraces and breeding lines were separated more efficiently. However, the sorghum accessions were not clearly separated according to their origin, as has been demonstrated for barley (Ordon et al., 1997). Groups of breeding lines and landraces include accessions of distant origin. Ayana et al. (2000) reported a weak differentiation of sorghum accessions according to both agro-ecological adaptation zones and regions of origin and supposed out-crossing and seed movement to be the reason for this. Most accessions in the present study can be assigned to the same agro-ecological zone, but the cultivation of varieties for different purposes, such as porridge and beer sorghums, may blur regional differentiations. Information on genetic relatedness in connection with knowledge on agronomic traits may have an impact on sorghum breeding. A breeding strategy is to choose well-adapted parents that possess many random genetic differences in the hope of an increased number of transgressive recombinants (cf. Tinker et al., 1993). Detailed genetic similarity estimates based on molecular markers are well suited for the selection of parental genotypes, since information about the number of segregating loci is provided.

The variance of G(RP) depends on the number of chromosomes and on the number of crossing-over events per bivalent, i.e. on the number of independently inherited chromosome segments. The greater the number of chromosomes and crossing-over events, the lower the variability of G(RP) and the incidence of extreme values close to 0.5 and 1.0. The present study elucidates the possibility to select single plants in BC_1 containing a G(RP) close to that expected in BC_2 or BC_3. Selection in BC_1 instead of BC_2 or BC_3 results in significant reductions in cost and time. The negative correlation between G(RP) and yielding ability under rainfed conditions in (LS3728 × IS4163) × IS4163 can be explained by heterosis effects. The degree of heterozygosity of BC_1 plants with extremely low G(RP)s is close to that of the F_1; the heterozygosity of the F_1 corresponds to the parental genetic distance. On average 50% of the heterozygosity in BC_1 is retained in BC_1S_1. It was expected that the selection of plants according to G(RP) in the BC_1 of (NP4453 × IS4394) × IS4394 might result in pre-selection for yielding ability, since the population is composed of a high-yielding cultivar and a landrace with poor yielding ability. Positive impacts of a certain degree of heterozygosity and low-input suitability contributed by the landrace may have led to the superior yields of some BC_1S_1 families with intermediate G(RP). A significant but weak correlation (R=0.42) between parental genetic distance and hybrid yields was observed by Jordan et al. (2003). Low correlations between genome-wide estimates of G(RP) and yields under low-input conditions may be due to the fact that genes or QTLs affecting yield and/or low-input suitability are unlikely to be evenly distributed over the genome.

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References

Aboubacar, A., Kirleis, A. W., Oumarou, M. (1999): Important sensory attributes affecting consumer acceptance of sorghum porridge in West Africa as related to quality tests. *J. Cereal Sci.*, **30**, 217–225.

Abu Assar, A. H., Uptmoor, R., Wagner, C., Abdelmula, A. A., Salih, M., Ordon, F., Friedt, W. (2005): Genetic variation in sorghum (*Sorghum bicolor* (L) Moench) germplasm from Sudan, ICRISAT and USA assessed with simple sequence repeats. *Crop Sci.*, **45**, 1636–1644.

Ahmed, M. M., Sanders, J. H., Nell, W. T. (2000): New sorghum and millet cultivar introduction in Sub-Saharan Africa: impacts and research agenda. *Agric. Systems*, **64**, 55–65.

Ajmone Marsan, P., Castiglioni, P., Fusari, F., Kuiper, M., Motto, M. (1998): Genetic diversity and its relationship to hybrid performance in maize as revealed by RFLP and AFLP markers. *Theor. Appl. Genet.*, **96**, 219–227.

Ayana, A., Bryngelsson, T., Bekele, E. (2000): Genetic variation of Ethiopian and Eritrean sorghum (*Sorghum bicolor* (L.) Moench) germplasm assessed by random amplified polymorphic DNA (RAPD). *Genet. Res. Crop Evol.*, **47**, 471–482.

Bohn, M., Utz, F. H., Melchinger, A. E. (1999): Genetic similarities among winter wheat cultivars determined on the basis of RFLPs, AFLPs and SSRs and their use for predicting progeny variance. *Crop Sci.*, **39**, 228–237.

Ceccarelli, S. (1994): Specific adaptation and breeding for marginal conditions. *Euphytica*, **77**, 205–219.

Djè, Y., Forcioli, D., Ater, M., Lefèbvre, C., Vekemans, X. (1999): Assessing population genetic structure of sorghum landraces from North-Western Morocco using allozyme and microsatellite markers. *Theor. Appl. Genet.*, **99**, 157–163.

Djè, Y., Heurtz, M., Lefèbvre, C., Vekemans, X. (2000): Assessment of genetic diversity within and among germplasm accessions in cultivated sorghum using microsatellite markers. *Theor. Appl. Genet.*, **100**, 918–925.

Goldstein, D. B., Ruíz-Linares, A., Cavalli-Sforza, L. L., Feldmann, M. W. (1995): An evaluation of genetic distances for use with microsatellite loci. *Genetics*, **139**, 463–471.

Grenier, C., Deu, M., Kresovich, S., Bramel-Cox, P. J., Hamon, P. (2000): Assessment of genetic diversity in three subsets constituted from the ICRISAT sorghum collection using random vs non-random sampling procedures B. Using molecular markers. *Theor. Appl. Genet.,* **101**, 197–202.

Haussmann, B. I. G., Obilana, A. B., Ayiecho, P. O., Blum, A., Schipprack, W., Geiger, H. H. (2000): Yield and yield stability of four population types of grain sorghum in a semi arid area of Kenya. *Crop Sci.,* **40**, 319–329.

Jordan, D. R., Tao, Y., Godwin, I. D., Henzell, R. G., Cooper, M., McIntyre, C. L. (2003): Prediction of hybrid performance in grain sorghum using RFLP markers. *Theor. Appl. Genet.,* **106**, 559–567.

Lynch, M., Milligan, B. G. (1994): Analysis of population genetic structure with RAPD markers. *Mol. Ecol.,* **3**, 91–99.

Nei, M. (1973): Analysis of gene diversity in subdivided populations. *Proc. Natl. Acad. Sci. USA,* **70**, 3321–3323.

Nei, M., Li, W. H. (1979): Mathematical model for studying genetic variation in terms of restriction endonucleasis. *Proc. Natl. Acad. Sci. USA,* **76**, 5269–5273.

Ordon, F., Schimann, A., Friedt, W. (1997): Assessment of the genetic relatedness of barley accessions (*Hordeum vulgare* s.l.) resistant to soil-borne mosaic-inducing viruses (BaMMV, BaYMV, BaYMV-2) using RAPDs. *Theor. Appl. Genet.,* **94**, 325–330.

Russell, J. R., Fuller, J. D., Macaulay, M., Hatz, B. G., Jahoor, A., Powell, W., Waugh, R. (1997): Direct comparison of levels of genetic variation among barley accessions detected by RFLPs, AFLPs, SSRs and RAPDs. *Theor. Appl. Genet.,* **95**, 714–722.

Simioniuc, D., Uptmoor, R., Friedt, W., Ordon, W. (2002): Genetic diversity and relationships among pea cultivars (*Pisum sativum* L.) revealed by RAPDs and AFLPs. *Plant Breeding,* **121**, 429–435.

Tanksley, S. D., McCouch, R. (1997): Seed banks and molecular maps: Unlocking genetic potential from the wild. *Science,* **277**, 1063–1066.

Tinker, N. A., Fortin, M. G., Mather, D. E. (1993): Random amplified polymorphic DNA and pedigree relationships in spring barley. *Theor. Appl. Genet.,* **85**, 976–984.

Toure, A., Traore, K., Bengaly, A., Scheuring, J. F., Rosenow, D. T., Rooney, L. W. (1998): The potential of local cultivars in sorghum improvement in Mali. *Afr. Crop Sci. J.,* **6**, 1–7.

Uptmoor, R., Wenzel, W. G., Friedt, W., Donaldson, G., Ayisi, K., Ordon, F. (2003): Comparative analysis on the genetic relatedness of *Sorghum bicolor* accessions from Southern Africa by RAPDs, AFLPs and SSRs. *Theor. Appl. Genet.,* **106**, 1316–1325.

Wenzel, W. G., Ayisi, K. K., Mogashoa, A., Donaldson, G., Mohammed, R., Uptmoor, R., Ordon, F., Friedt, W. (2001): Improved Sorghum varieties for smallholder farmers. *J. Appl. Botany,* **75**, 207–209.

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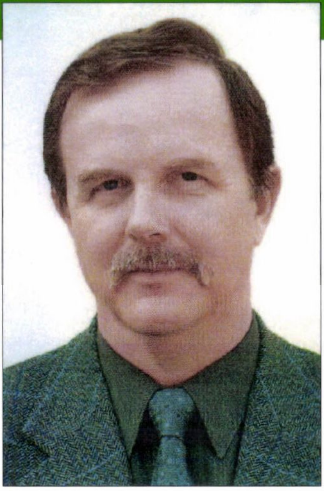

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HETEROSIS LEVEL OF MAIZE HYBRIDS DEVELOPED USING DNA TECHNOLOGIES

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The performance of maize hybrids developed on the basis of recombined inbred lines (RIL) selected from an F_2 hybrid population using marker-assisted selection (MAS) was studied. The task was to estimate the efficiency of DNA marker technology for intrapopulation selection and to study the performance of hybrids produced from marker-derived inbreds of the F_2 population (GK 26 × Mo 17). Two hundred RILs of marker origin were crossed with 3 unrelated testcross lines from the Lacaune, Mindszentpuszta and Reid heterotic groups. An effective marker test system and informative marker criteria were elaborated for increasing MAS effectiveness. A two-locus system on the basis of linked SSR markers proved to be the most effective. The genetic improvement effect (ΔG) of the C_1 population for plant productivity, plant height and grain length ranged from 9.1 to 16.1%, depending on the phenotypic trait and h^2 level. The best hybrids developed on the basis of RILs of marker origin outyielded the national check for grain yield by 6.8–7.6%.

Key words: marker-assisted selection, hybrid performance

Introduction

For a considerable length of time many plant breeders have been concerned that the genetic diversity of modern initial material has been decreasing at an alarming rate as a consequence of the modern breeding doctrine and agricultural practice (Lee, 1995). This idea is thoroughly confirmed by the situation in both European and American maize breeding practice. Many maize inbreds have been developed from a limited number of elite lines or from synthetics that are composed of elite lines. This practice heightens the risk of decreasing genetic diversity in the field of commercial maize production (Hallauer et al., 1988). One of the breeding approaches for simultaneously solving two tasks: widening genetic diversity and using it for line development in a hybrid breeding programme, is intra- and interpopulation selection. Until the past two decades selection gain and genetic diversity were evaluated mostly using phenotypic markers and the biometric analysis of quantitative variation (Hallauer and Miranda, 1982; Shivaji-Pandey, 1999). When it became possible to use the genotype information from molecular markers in breeding programmes a method of marker-assisted selection (MAS) was elaborated (Lande and Thompson, 1990) for utilizing the linkage disequilibrium between inbred line genomes.

The selection progress of MAS for intrapopulation improvement was first investigated in the basic analytic approach of Lande and Thompson (1990). Then this approach was extended and discussed for cases of broad (Stam, 1994) and finite population sizes (Hospital et al., 1997; Moreau et al., 1997). The MAS efficiency problem has been studied broadly at the level of computer simulations (Zhang and Smith, 1993; Gimelfarb and Lande, 1995; Wittaker et al., 1995; Hospital et al., 1997). The application of MAS for crop improvement in practical breeding programmes is still very limited. Testcross performance in MAS was studied for a maize population (Stromberg et al., 1994). Later the practical results of MAS-hybrid population improvement were presented for a variety of important agronomic traits (Domenjuk et al., 2002a, b; Sivolap et al., 2003).

The objectives of this study were to: (i) elaborate a tester system of markers for MAS improvement of a population; (ii) estimate the genetic parameters of a population improved by MAS for important agronomic traits; (iii) evaluate the hybrid performance of half-sibs developed on the basis of RILs MAS-origin.

Materials and methods

Plant material

An F_2 hybrid population was obtained by crossing GK 26 (Iodent) and Mo 17 (Lancaster) inbred lines, with the subsequent sibbing of the F1 hybrid. Subsequent selection was made from the F2 (GK 26 × Mo 17) for 17 agronomically or information important traits (Table 1) by selfing and applying the SSR and ISSR marker techniques. A medium population size (N) was used that included 200 plants for the estimation of trait values and polymorphism genotyping.

Selection procedure and evaluation trials

The intensivity of selection in the F_3–F_4 generations was 10%. On the basis of the best F_4 genotypes for three important traits, plant productivity (PP), plant height (PH) and kernel length (KL), two populations were formed: the population of the first selection cycle (C_{m1}), which was genetically improved by the MAS procedure, and the unselected but mated initial F_4 population. On the basis of the C_{m1} population, 180 genetically improved inbred recombined lines of I_6 were made. They were test-crossed to three elite lines, Od 308, Od 221 and Od 329, belonging to the Mindszentpuszta, Lacaune and Reid heterotic groups, respectively. The 480 half-sibs produced were studied in preliminary tests in two ecological zones (steppe, forest steppe). The ten best hybrids were evaluated in ecological trials in 2005, which covered eight locations in the main climatic zones of Ukraine. The set of testcrosses was grown in randomized complete blocks with three replications. The selection criteria were: grain yield, adjusted to 14% grain moisture, grain moisture at harvest, and lodging, estimated as the percentage of plants showing either root or stalk lodging.

Table 1
Statistical parameters of 17 phenotypic traits for the population (GK 26 × Mo 17)F_2 and its parental lines

Trait	Mean			s		
	GK 26	Mo 17	F_2	GK 26	Mo 17	F_2
Plant height, cm	158	173	186	6.1	6.6	16.5
Ear insertion height, cm	62	72	73	4.6	6.6	10.3
Lodging	0	0	2.1	0	0	1.9
Ear leaf width, cm	7	8	8	0.5	1.1	1.2
No. of tassel branches	17	7	8	2.0	1.5	1.8
Tassel length, cm	34	34	36	1.8	1.4	3.9
No. of kernel rows	12	8	12	0.7	1.0	1.1
100-kernel weight, g	30	37	27	2.0	3.6	5.8
Kernel length, cm	2	1.5	1.5	0.3	0.2	0.2
Ear diameter, cm	3	3	4	0.2	0.3	0.4
Ear length, cm	12	14	16	1.1	1.9	1.6
Ear leaf length, cm	55	70	66	1.0	1.6	5.5
Protein content, %	12	10	10	1.0	0.7	0.9
Starch content, %	53	59	56	2.0	4.5	4.0
Oil content, %	3.0	3.0	3.0	0.6	0.3	1.3
Grain yield per plant, g	44	54	88	11.2	19.0	20.6

DNA extraction

Total genomic DNA was extracted from the young leaves of each F_2 and F_3 plant according to the procedure of Ribaut et al. (1997) with some modifications: in order to improve the purity of the genomic DNA, polyvinylpyrrolidone (PVP) and two washes with buffer (76% C_2H_5OH + 0.2 M CH_3COOH; 76% C_2H_5OH + 10 mM CH_3COONH_4) were applied.

SSR and ISSR genotyping

The PCR reaction mix contained: 50 mM KCl, 20 mM tris-HCl (pH 8.4 at 25°C), 2–4 mM $MgCl_2$ (for ISSR and SSR primers, respectively), 0.01% Tween-20, 0.15 mM each dNTP, 0.2 μM primers, 10–20 ng DNA, 0.8–1 U Tag-polymerase (total volume 20 μl).

The SSR and ISSR primers were selected from http://www.maizegdb.org. A total of 20 SSR primers were used but polymorphic amplification products were only produced for a quarter of them. Eighteen ISSR primers were studied and polymorphism was detected by three of them. The MJ Research (PTC-200) system was employed for PCR. Temperature conditions: denaturation: 94°C for 40 s (SSR loci); elongation: 72°C for 2 min; 30–35 cycles; last elongation: 72°C for 5 min.

The PCR products were separated on 2–4% agarose. To increase the quality of resolution 6–10% polyacrylamide gels were used (for ISSR and SSR fragments, respectively). Separated fragments on the gels were sized by reference to the molecular weight marker pUC18/MspI (Fermentas). The data were recorded with the video system VDS (Pharmacia Biotech) and calculated with the Image Master 1d Elite program.

QTL analysis

A phenotypic database was compikled from 200 plants based on the segregation of 17 traits in the F_2 population. They were genotyped and the polymorphic amplicon inheritance was traced. For each marker allele of each SSR and ISSR locus the selective weight was determined as a definite trait mean for all plants in the population that had the given allele. Loci exhibiting significant differences ($P<0.01$) between phenotypic classes for corresponding alleles were considered to be markers. QTL analysis was performed on the basis of the Tanksley algorithm (1993) using the programs Gene-Stat and Statistica. Linkage analysis on the set of F_2 population plants was performed using the F_2 model of mapmaker/exp 3.0 (Lander et al., 1987). The data of hybrid trials were analysed using the Statistica program package.

Results

It was revealed that the most important traits were marked by 2–4 polymorphic loci. For instance four marker loci were associated with the plant productivity and kernel length, three with ear diameter and two with 100-kernel weight. No markers were found for row number, ear length or grain moisture.

Simulation made it possible to formulate the most important criteria for a marker selection system: (i) the best marker type is SSR, as only for co-dominant markers can all the locus alleles be visualized, (ii) co-dominant and dominant markers can be combined provided the marker alleles are appopriate, (iii) a locus has the best marker potential when it is in the homozygous condition, (iv) linked marker loci are a good tool for a marker test system. Only markers that showed the best information potential in two adjacent F_2–F_3 generations were selected.

Marker-assisted selection was performed on three traits for which marker test systems (MTS) were formed. Samples that were linked with more than one marker were retained in the F_3: plant height – two homozygous SSR markers, nc 030-108 (marker name with the molecular weight of a polymorphic product) and phi 061-88; kernel length – three homozygous ISSR markers, isp5-950, isp5-378 and isp13-525; plant productivity – two homozygous markers, nc 030-108 and phi 061-88, and one ISSR marker, isp5-950. The loci nc 030-108 and isp5-950 were linked. The intensity and selection differential level of marker-assisted selection in F_3 for the three aforenamed traits are shown in Table 2. The marker-selected subpopulation F_3 significantly exceeded ($P<0.01$–$P<0.001$) the initial F_3 for both the mean and maximum trait values. An especially high Sd (16.8 units) was observed for plant productivity. The maximum trait values of the subpopulation were found to be significantly better than for the initial population. For instance, the best grain productivity in the initial population was 59.0 g/plant, as against 76.1 g/plant in the selected subpopulation.

It should be emphasized that the marker test systems for plant productivity and plant height were established using the same markers. The initial population and selected subpopulation seed were sown in the same year. Both the unselected population (C_o) and the RIL-population of the first cycle of marker-assisted selection (C_{m1}) were analysed for trait performance (Table 3). The C_{m1} population outperformed C_o in terms of selection progress and genetic gain and in fact for all estimated traits. Especially significant genetic progress was observed for plant height (9.1%) and plant productivity (16.1%). The selection response for kernel height proved to be non-significant (Table 3).

Table 2

Selection differential between the mean of the initial (unselected) population F_3 and the selected subpopulation F_3 for selection intensity 15% and population size N = 200

Statistical index	F_3 populations		Selection differential
	Initial	Subpopulation	
	Plant height, cm, MTS: nc 030, phi 061		
X±Sx	142.7±1.65	149.9±1.17	7.2^{++}
S	16.2	15.2	
Lim	135–151	144–159	
	Kernel length, cm, MTS: isp5 – 950, isp5 – 378, isp13 – 525		
X±Sx	1.34±0.03	1.48±0.03	0.14^{++}
S	0.27	0.23	
Lim	1.2–1.5	1.4–1.6	
	Plant productivity, g, MTS: nc 030, phi 061		
X±Sx	40.1±3.36	56.9±2.80	16.8^{+++}
S	31.7	29.6	
Lim	30.2–59.0	44.0–76.1	

$^{++}$, $^{+++}$ - significant at the P<0.01 and P<0.001 level, respectively; MTS - marker test system

Table 3

Population response (R) and genetic gain (ρG) after 1st cycle of marker-assisted selection for the traits plant height, grain length and plant productivity

Statistical index	Population		Population response	
	Co	C1-m	R	ρG, %
	Plant height, cm, MTS: nc 030, phi 061			
X±Sx	123±1.65	135.1±1.23	12.1^{+++}	9.1
S	16.2	13.6		
Lim	115–131	130–140		
	Grain lenght, cm, MTS: isp5 – 950, isp5 – 378, isp13 – 525			
X±Sx	1.4±0.03	1.46±0.02	0.06	3.9
S	0.27	0.23		
Lim	1.2–1.5	1.4–1.5		
	Plant productivity, g, MTS: nc 030, phi 061			
X±Sx	48.4±3.36	57.2±1.37	8.8^{++}	16.1
S	29.6	21.9		
Lim	30.2–59.0	47.0–64.3		

$^{++}$, $^{+++}$ - significant at the P<0.01 and P<0.001 level, respectively. MTS - marker test system

After four cycles of inbreeding on the C_{m1} population, nearly 200 RILs were derived for producing testcrosses with three testers and their heterotic potential was estimated. A preliminary study on 480 testcrosses showed the high level of their grain performance under arid and favourable conditions. This was confirmed by the data of ecological trials on ten outstanding hybrids tested in 2005 in eight locations covering the main climatic zones of Ukraine: (1) Steppe – Dachnaya Experimental Farm of the Plant Breeding and Genetics Institute, Odessa region; (2) Cereal Crops Institute, Dnepropetrovsk region; (3)

Sinelnikovo Breeding Station, Dnepropetrovsk region; (4) Mais Company, Dnepropetrovsk region; (5) Institute of Agro-Industrial Production, Lugansk region; (6) Agriculture Institute of Southern regions, Kherson region; (7) Institute of Agro-Industrial Production of the Forest-Steppe zone, Cherkass region and (8) Agricultural college, Ternopol region.

The analysis for grain yield revealed highly significant variation both among testcross hybrids of marker origin and between hybrid means at different locations (Table 4). The hybrid that performed best among the testcross means in the Steppe zone was OBC 377, the grain yield of which exceeded both the check and the other testcrosses. However, the hybrid ranking in the Forest – Steppe zone changed and the leaders for grain yield proved to be OBC 379, OBC 383, OBC 380 and OBS 381. They significantly exceeded the check, and the testcross OBC 379 even surpassed OBC 377. Nevertheless, averaged over all locations in both zones the best testcross hybrid was OBC 377, which significantly exceeded the check in the Forest – Steppe zone and on average over all testing locations. The maximum yield was exhibited by two hybrids, OBC 386 under semi-irrigation conditions (10.61 t/ha) and OBC 383 (10.38 t/ha) under dry conditions. The most competitive heterosis level and the best adaptability was exhibited by OBC 377 for all locations and in the Steppe region (1.6%). A very high heterosis level was also displayed in the Forest-Steppe region by single crosses OBC 379 (26.2%), OBC 383 (21.3%) and OBC 380 (20.1%), which showed very good results throughout the zones. A preliminary study in 2004 and ecological testing in 2005 resulted in submitting the best single cross, OBC 377, for state hybrid trials in 2006. The full characterization of its agronomic traits is presented in Table 5. The new hybrid significantly surpassed one of the best national checks in Ukraine, Dar 347, not only for potential productivity but alsofor such valuable characters as harvest grain moisture, lodging and drought resistance, and tolerance to loose smut and stalk rot infection.

Table 4

Average grain yield of the best testcrosses of ten RILs of marker origin from the C_{m1} population and the inbred tester Od 308 MR, evaluated at eight locations in the Steppe and Forest-Steppe regions

Testcross hybrid	Steppe locations									Forest-steppe locations					Mean		
	1	2	3	4	5	6	M***	D**		7	8	M***	D		t ha^{-1}	D	
								t ha^{-1}	%				t ha^{-1}	%		t ha^{-1}	%
OBC 377	3.12	8.66	5.24	6.37	4.01	9.92	6.22	0.10	1.6	9.29	8.84	9.06	1.68	18.5	6.93	0.5	7.2
OBC 378	2.78	7.96	4.81	6.18	4.67	8.84	5.87	–0.25	–4.25	8.71	8.47	8.59	1.21	14.1	6.55	0.12	1.8
OBC 379	3.24	7.39	4.10	6.73	4.31	7.60	5.56	–0.56	–10.1	10.23	9.76	10.0	2.62	36.2	6.67	0.24	3.6
OBC 380	3.50	8.86	4.16	6.63	4.41	7.95	5.92	–0.20	–3.4	8.96	9.52	9.24	1.86	20.1	6.75	0.32	4.7
OBC 381	3.23	6.91	3.94	5.97	4.42	9.25	5.64	–0.48	–8.5	9.55	8.91	9.23	1.85	20.0	6.53	0.1	1.5
OBC 382	2.87	9.12	3.40	6.11	4.79	7.71	5.67	–0.45	–7.9	8.68	7.07	7.88	0.50	6.3	6.22	–0.21	–3.4
OBC 383	2.80	8.52	4.12	6.86	5.06	8.95	6.05	–0.07	–1.15	10.38	8.38	9.38	2.0	21.3	6.88	0.45	6.5
OBC 384	2.53	7.61	3.91	6.20	5.75	9.25	5.88	–0.24	–4.1	9.58	7.95	8.76	1.38	15.8	6.60	0.17	2.6
OBC 385	3.06	9.08	3.10	6.79	4.53	9.00	5.93	–0.19	–3.2	9.24	7.06	8.15	0.77	9.4	6.48	00.5	0.8
OBC 386	3.11	8.06	3.46	5.69	5.28	10.61	6.04	–0.08	–1.3	9.03	8.15	8.59	1.21	14.1	6.67	0.24	3.6
Dar 347*	3.8	8.86	5.58	5.43	5.39	8.39	6.12	0	0	7.64	7.11	7.38	0	0	6.43	0	0
LSD$_{0.05}$	0.18	0.45	0.51	0.26	0.53	0.43		0.25		0.53	0.51		0.58			0.32	

*check; ** Deviation from the check; ***:Mean

Table 5
Average performance of eleven characters for the best single cross of marker origin (OBC 377) and the check (Dar 347)

Character	Hybrid	
	OBC 377	Dar 347 (check)
Maximum grain yield, t/ha	9.92	8.39
Harvest grain moisture, %	22.7	26.4
Season period, days	118–120	116–119
Plant height, cm	173–175	175–178
Ear insertion height, cm	60-65	61-66
Weight of 1000 grains, g	270–280	340–360
Lodging resistance, note	9	8
Drought resistance, note	9	7.5
Loose smut infection, %	7.0	9.0
Common smut infection, %	3.6	2.4
Stalk rot infection, %	9.1	12.3

Discussion

It is clear from the results of the present study as well as from other previous research, that marker-assisted selection (MAS) can be successfully utilized for the genetic improvement of the population. The genetic gain for plant height and plant productivity in this study after the first cycle of selection amounted to 9.1 and 16.1%, respectively, for the small population size (N=200), with heritability levels of $h^2=0.29$ and 0.37, respectively. These data are consistent with the results of Hospital et al. (1997), Moreau et al. (1997) and Moreno-Gonzales (1999), who used computer simulation to show the dependence of MAS gains on the relationships of such parameters as population size (including small size, N=200), heritability level and the error-type risk of the regression in the first generations. These problems were discussed earlier by Lande and Thompson (1990), Zhang and Smith (1993) and Gimelfarb and Lande (1995).

The application of crop improvement by MAS in practical breeding is still very limited. Stromberg et al. (1994) reported the results of comparing of conventional testcross performance with testcrosses produced on the basis of MAS progenies of a maize population. The present results showed that a maize population can be genetically improved by one cycle of marker-assisted selection for grain productivity (16.1%) and for other important traits. Highly productive hybrids can be developed from a population on the basis of MAS-improved recombinant inbred lines. In the present experiment the best hybrid of marker origin was significantly superior to the best conventional check for grain performance and for other important agronomic traits.

References

Domenjuk, V. P., Verbitskaja, T. G., Belousov, A. A., Sivolap, J. M. (2002a): Marker analysis of maize quantitative traits by ISSR–PCR. (Маркерный анализ количественных признаков кукурузы при помощи ISSR – ПЦР.) *Genetics*, **38**, 1370–1378.

Domenjuk, V. P., Belousov, A. A., Sivolap, J. M. (2002b): DNA-markering of quantitative traits of maize. (ДНК-маркирование количественных признаков кукурузы.) *Cytology and Genetics*, **36**, 6, 9–15.

Gimelfarb, A., Lande, R. (1995): Marker-assisted selection and marker-QTL associations in hybrid populations. *Theor. Appl. Genet.*, **91**, 522–528.

Hallauer, A. R., Miranda, J. B. (1982): *Quantitative Genetics in Maize Breeding*. Iowa State Univ. Press, Ames, 468 p.

Hallauer, A. R., Russell, W. A., Lamkey, K. R. (1988): Corn breeding. pp. 463–564. In: Sprague, G. F., Dudley, J. W. (eds.), *Corn and Corn Improvement*. 3rd ed. Agron. Monogr., 18, ASA, CSSA and SSSA, Madison, WI.

Hospital, F., Moreau, L., Lacoudre, F., Charcosset, A., Gallais, A. (1997): More on the efficiency of marker-assisted selection. *Theor. Appl. Genet.*, **95**, 1181–1189.

Lande, R., Thompson, R. (1990): Efficiency of marker-assisted selection in the improvement of quantitative traits. *Genetics*, **124**, 743–756.

Lander, E., Green, P., Abrahamson, J., Barlow, A., Daly, M., Lincoln, S., Newburg, L. (1987): MAPMAKER: An interactive computer package for constructing primary genetic linkage maps of experimental and natural population. *Genomics*, **1**, 174–181.

Lee, M. (1995): DNA markers and plant breeding programs. *Adv. Agron.*, **35**, 265–344.

Moreau, L., Charcosset, A., Hospital, F., Gallais, A. (1997): Marker-assisted selection efficiency in populations of finite size. *Genetics*, **148**, 1353–1365.

Moreno-Gonzales, J. (1999): Molecular marker and heterosis. pp. 257–268. In: Melchinger, A. E., Coors, J. G. (eds.), *The Genetics and Exploitation of Heterosis in Crops*. Proceedings of an international symposium, CIMMYT, Mexico City, Mexico, 17–22 August, 1997.

Ribaut, J.-M., Hu, X., Hoisington, D., Gonzales, D. (1997): Use of STSs and SSRs as rapid and reliable preselection tools in a marker-assisted selection-backcross scheme. *Plant Molecular Biology Reporter*, **15**, 154–162.

Shivaji-Pandey (1999): Genetic diversity and heterosis. pp. 99–118. In: Melchinger, A. E., Coors, J. G. (eds.) *The Genetics and Exploitation of Heterosis in Crops.* Proceedings of an international symposium, CIMMYT, Mexico City, Mexico, 17–22 August, 1997.

Sivolap, J. M., Sokolov, V. M., Belousov, A. A., Domenjuk, V. P. (2003): Genetical improvement of maize populations by DNA-marker-assisted selection of QTLs. (Генетичне поліпшення популяцій кукурудзи шляхом добору за ДНК-маркерами QTLs.) *Methodical Recommendations*. Odessa, Ukraine, 12 p.

Stam, P. (1994): Marker-assisted breeding. In: van Ooijen, J. W., Jansen, J. (eds.), *Biometrics in Plant Breeding: Applications of Molecular Markers*. Proc. of the 9th meeting of the EUCARPIA section of biometrics in plant breeding. 6–8 July 1994, Wageningen, the Netherlands.

Stromberg, L. D., Dudley, J. W., Rufener, G. K. (1994): Comparing conventional early generation selection with molecular marker assisted selection in maize. *Crop Sci.*, **34**, 1221–1225.

Tanksley, S. (1993): Mapping polygenes. *Annu. Rev. Genet.,* **27**, 205–233.

Wittaker, J. C., Curnow, R. N., Haley, C. S., Thompson, R. (1995): Using marker-maps in marker-assisted selection. *Genet. Res.*, **66**, 255–265.

Zhang, W., Smith, C. (1993): Simulation of marker-assisted selection utilizing linkage disequilibrium: the effects of several additional factors. *Theor. Appl. Genet.*, **86**, 492–496.

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ADAPTATION OF MAIZE LINES AND HYBRIDS TO ABIOTIC/BIOTIC STRESSES

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The resistance of maize inbred lines and their hybrids to Western Corn Rootworm was investigated in a 4 × 4 full diallel system. The most tolerant line against WCR larvae was the inbred line P26.

Four maize inbred lines and their 12 normal and reciprocal crosses were investigated for resistance to *Fusarium* ssp. and European Corn Borer under natural conditions in four replications in 1998–2000. The highest GCA values were found for the inbred lines P26 and P50.

Studies were also made to determine the optimum concentration of imidazolinone in the selective medium for the detection of resistant cell lines originating from homozygous genotypes produced by irradiation.

Keywords: *Fusarium* ssp., *Zea mays* L., European Corn Borer, Western Corn Rootworm, imidazolinone resistance

Introduction

Abel et al. (2000) found that the development of resistant plants was a promising method for controlling the most damaging insect pest of maize, *Diabrotica virgifera virgifera* LeConte. Maize lines derived from a backcross breeding programme were used to transfer resistance into inbred lines.

Urias-Lopez and Meinke (2001) suggested that maize hybrids may differ inherently in their ability to tolerate rootworm injury and partition biomass in response to injury and other stresses.

Urias-Lopez et al. (2000) found that there may be a common negative photosynthetic response within maize to larval injury. Transient reductions in photosynthetic rate occurred in rootworm-infested maize, which after a lag period, led to significant reductions in plant height.

Moeser and Vidal (2004) studied the behaviour of larvae of *Diabrotica virgifera virgifera* LeConte (Chrysomelidae, Galerucinae) on different European maize lines and observed great variability among the genotypes.

Fusarium species reduce the yield and seed quality of maize both in Hungary and worldwide by causing stalk, root and ear rot. Damage by *Fusarium* sp. depends principally on the cultivation technique and weather factors (e.g. insect control, hail, etc.) and on the maize genotype.

In many cases recently developed herbicides with great effectiveness, a particular spectrum and quick degradability have an adverse effect on the crop.

Materials and methods

The aim of the study was to determine the fusarium ear rot and European Corn Borer resistance of the inbred lines and hybrids in our breeding programme. Four maize inbred lines (P14, P26, P50 and P61) and their 12 normal and reciprocal crosses were investigated for resistance to *Fusarium* ssp. and European Corn Borer under natural conditions in four replications in 1998–2000.

The general and specific combining ability for fusarium ear rot and ECB resistance were estimated by applying a full diallel system according to the modified Griffing's method 1. The analysis of combining ability revealed that significant differences existed between the maize inbred lines and hybrids with respect to general (GCA) and specific (SCA) combining ability for resistance to fusarium ear rot and ECB.

The resistance/tolerance of the maize inbred lines and their hybrids was also investigated to Western Corn Rootworm using a 4 × 4 full diallel system.

The experiments were set up in a lime-coated chernozem soil near Debrecen with the same technology and under the same ecological conditions for 3 years (2002, 2003, 2004). The data (larval damage on the Iowa scale, number of adults/plant, lodging %, yield loss %) were analysed using an improved variant of DAS (Diallel Analysis and Simulation).

The optimum imidazolinone concentration in the selective medium was determined for the detection of resistant cell lines originating from homozygous genotypes produced by irradiation in the Department of Plant Genetics and Breeding, Centre for Agricultural Sciences, University of Debrecen.

After two-step *in vivo* selection, the maize lines were selected using the 1% concentration recommended for imidazolinone herbicides (4 l/ha ESCORT™). Based on their response to herbicide, five inbred lines (176/F, 241/F, 271/F, 378/F, 416/F) were chosen for callus induction.

The best medium for the multiplication and differentiation of plant cells *in vitro* was MS (Murashige-Skoog) supplemented with 12.5 μM l^{-1} 2,4-D (2,4-dichloro-phenoxy-acetic acid). The *in vitro* experiments were repeated with a 0.012% imidazolinone concentration.

Results and discussion

The resistance/tolerance of maize inbred lines and their hybrids to Western Corn Rootworm was investigated using a 4×4 full diallel system. The data were analysed using an improved variant of Diallel Analysis and Simulation. The most tolerant line against WCR larvae (*Diabrotica virgifera*) was the inbred line P26, where the larval damage was 0.5, 1.5 and 2.6 on the Iowa scale in the consecutive years (Fig. 1, Table 1).

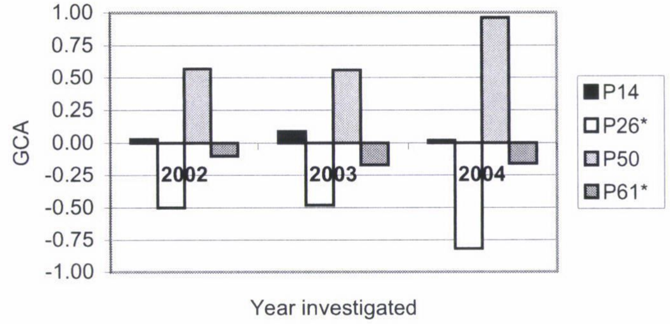

Fig. 1. Evaluation of general combining ability for larval damage based on the Iowa scale (*State registered lines)

Table 1
Specific combining ability (above diagonal) and reciprocal effect (below diagonal) based on larval damage in maize

Female lines	Male lines											
	2002				2003				2004			
	P14	P26	P50	P61	P14	P26	P50	P61	P14	P26	P50	P61
P14		–0.32	0.1	0.2		-0.13	0.244	0.105		–0.1	0.2	0.11
P26	0.05		0.081	0.031	–0.1		–0.19	0.07	–0.05		–0.16	–0.02
P50	–0.125	0.05		0.16	–0.025	0.201		–0.006	0.05	–0.175		0.429
P61	–0.1	–0.75	–0.06		0.15	0.027	–0.4		–0.17	0.16	0.12	

R^2 (2002) = 0.9942; R^2 (2003) = 0.9744; R^2 (2004) = 0.9963

The number of imagos per plant was lowest in line P26, which proved to be tolerant to Western Corn Rootworm. The GCA for lodging resistance was similar to those for larval damage and adult number (Fig. 2, Table 2).

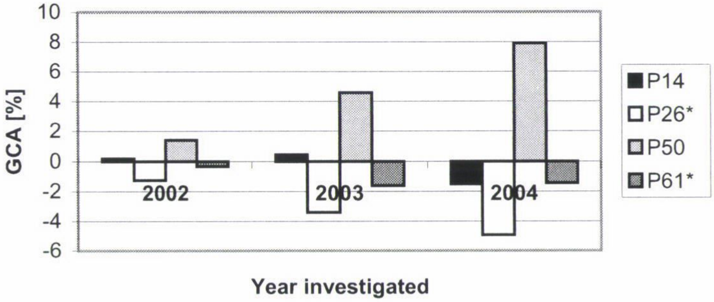

Fig. 2. GCA values based on lodging in maize lines (*State registered lines)

Table 2

Specific combining ability (above diagonal) and reciprocal effect (below diagonal) values based on lodging in maize

Female lines	Male lines											
	2002				2003				2004			
	P14	P26	P50	P61	P14	P26	P50	P61	P14	P26	P50	P61
P14		–0.33	0.44	0.21		–0.09	1.34	0.41		0.41	–2.29	0.42
P26	0.25		–0.51	0.29	0.6		–0.79	1.82	–0.35		–2.87	1.18
P50	0.4	0.45		0.56	0.01	–0.9		–0.79	–0.3	–1.5		1.18
P61	–0.3	–0.25	–0.2		–0.9	0.45	9.0		0.85	0.01	5.15	

R^2 (2002) = 0.9935; R^2 (2003) = 0.9952; R^2 (2004) = 0.9129

Line P26 decreased the yield loss in its cross combinations to a significant extent (Fig. 3, Table 3).

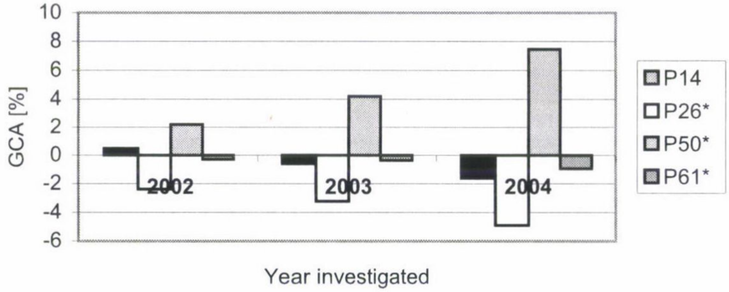

Fig. 3. Evaluation of general combining ability based on yield loss in maize (*State registered lines)

Table 3

Specific combining ability (above diagonal) and reciprocal effect (below diagonal) based on yield loss in maize lines and their hybrids

Female lines	Male lines											
	2002				2003				2004			
	P14	P26	P50	P61	P14	P26	P50	P61	P14	P26	P50	P61
P14		–1.03	0.11	0.04		0.38	–0.06	0.04		0.53	–2.58	0.02
P26	–0.50		–0.11	0.89	0.8		–2.87	0.89	–0.85		–4.43	1.09
P50	–0.05	0.16		0.63	–1.0	–1.2		0.63	–1.61	0.48		1.33
P61	–0.39	–0.23	–4.44		–1.16	–1.19	–0.61		–1.5	–0.6	–0.01	

R^2 (2002) = 0.9932; R^2 (2003) = 0.9954; R^2 (2004) = 0.9981

On the basis of the results it was concluded that the most efficient control of Western Corn Rootworm (*Diabrotica virgifera*) could be achieved by growing hybrids developed using resistant lines.

The resistance of four maize inbred lines (P14, P26, P50 and P61) and their 12 normal and reciprocal crosses to *Fusarium* ssp. and European Corn Borer was investigated under natural conditions in four replications in 1998–2000. The general and specific combining ability for fusarium ear rot and ECB resistance were estimated by applying a full diallel system according to the modified Griffing's method 1. The analysis of combining ability revealed that significant differences existed between maize inbred lines and their hybrids for general (GCA) and specific (SCA) combining ability for resistance to fusarium ear rot and ECB (Tables 4, 5 and 6).

Table 4

Evaluation of general (GCA) and specific (SCA) combining ability and reciprocal effect (RE) based on infection with European Corn Borer in maize lines and their hybrids (1998)

Female lines	Male lines			
	P14	P26	P50	P61
P14	1.89 GCA	2.11	–1.57	0.02
P26[x]	0.38	–2.80 GCA	–0.85	0.08
P50	–0.76	0.06	3.09 GCA	–0.05
P61[x]	0.01	–0.51	0.33	–2.19 GCA

R^2= 0.9980; SCA – above diagonal; RE – below diagonal; [x]State registered lines

Table 5

Evaluation of general (GCA) and specific (SCA) combining ability and reciprocal effect (RE) based on infection with European Corn Borer in maize lines and their hybrids (1999)

Female lines	Male lines			
	P14	P26	P50	P61
P14	0.07 GCA	2.11	1.98	–0.20
P26[x]	–0.63	–3.33 GCA	1.46	–0.48
P50	–0.06	–0.03	0.12 GCA	–0.58
P61[x]	–0.70	–0.15	–0.55	3.12 GCA

R^2= 0.9888; SCA – above diameter; RE – below diagonal; [x]State registered lines

Table 6

Evaluation of general (GCA) and specific (SCA) combining ability and reciprocal effect (RE) based on infection with European Corn Borer in maize lines and their hybrids (2000)

Female lines	Male lines			
	P14	P26	P50	P61
P14	2.94 GCA	1.44	0.38	0.81
P26[x]	–0.31	–2.99 GCA	–0.80	–0.35
P50	–1.06	–0.28	2.16 GCA	1.35
P61[x]	–0.09	–0.01	0.01	–2.11 GCA

R^2= 0.9916; SCA – above diameter; RE – below diagonal; [x]State registered lines

The highest GCA values were found for the maize inbred lines P26 and P50. The estimated SCA values showed that P26×P50 and P14×P61 were good crosses for reducing damage. This information could be used to select maize hybrids with low susceptibility, which could be integrated into the breeding programme.

Tests were made to determine the optimum imidazolinone concentration in the selective medium for the detection of resistant cell lines originating from homozygous genotypes produced by irradiation. After two-step *in vivo* selection maize lines were selected using the 1% concentration recommended for imidazolinone herbicides (4 l/ha ESCORT™). Based on their response to herbicide five inbred lines (176/F, 241/F, 271/F, 378/F, 416/F) were chosen for callus induction. When using a medium containing 0.1% imidazolinone the callus weight showed no increase because of the sensitivity of the lines (Fig. 4). At a concentration of 0.01% imidazolinone the calli of lines 176/F and 378/F did not grow, but the callus weight increase of lines 271/F and 416/F was 14.41% and 16.61%, respectively, while line 241/F showed nearly the same weight increase as the control. The *in vitro* experiments were repeated with 0.012 %, imidazolinone concentration, which confirmed that lines 176/F and 378/F were sensitive to imidazolinone (Fig. 5). The fresh callus weight of lines 271/F and 416/F decreased as the imidazolinone concentration increased, while the inbred line 241/F showed the same increase as the control.

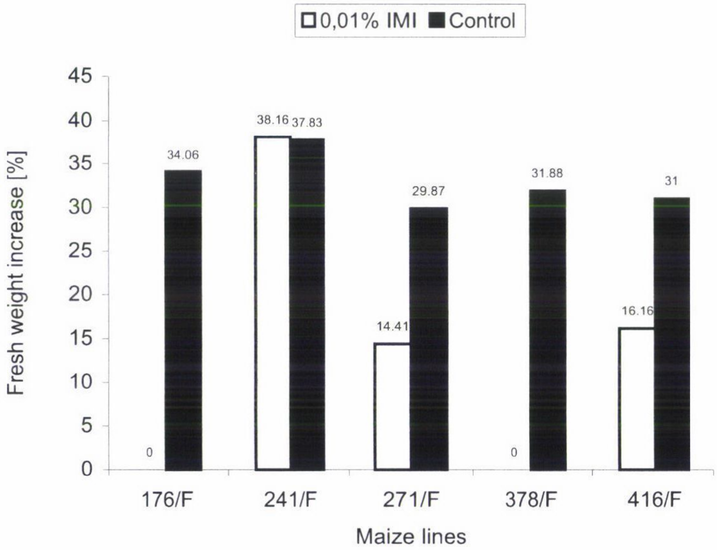

Fig. 4. Fresh weight increase of calli of maize lines on selective media supplemented with 0.01% imidazolinone (12.5 μM l^{-1} 2,4-D) and on the control medium (12.5 μM l^{-1} 2,4-D)

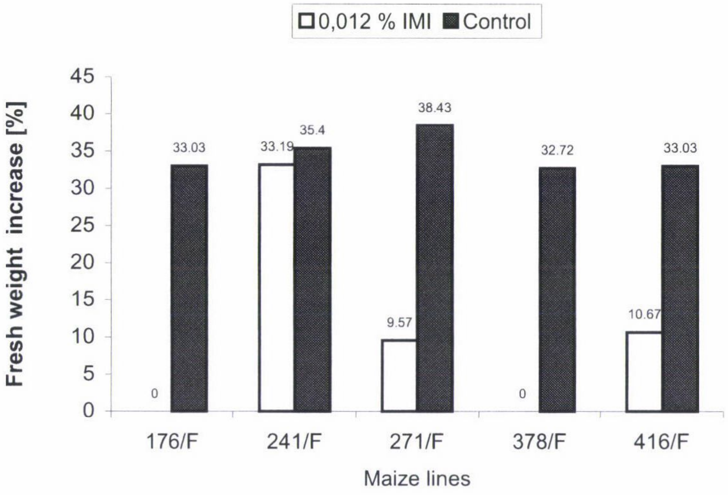

Fig. 5. Fresh weight increase of calli of maize lines on selective media supplemented with 0.012% imidazolinone (12.5 μM l^{-1} 2,4-D) and on the control medium (12.5 μM l^{-1} 2,4-D)

References

Abel, C. A., Berhov, M. A., Wilson, R. L., Binder, B. F., Hibbard, B. E. (2000): Evaluation of conventional resistance to European corn borer (*Lepidoptera: Crambidae*) and Western corn rootworm (*Coleoptera: Chrysomelidae*) in experimental maize lines developed from a backcross breeding program. *Journal of Economic Entomology*, **93**, 1814–1821.

Moeser, J., Vidal, S. (2004): Response of larvae of invasive maize pest *Diabrotica virgifera virgifera* (*Coleoptera: Chrysomelidae*) to carbon/nitrogen ratio and phytosterol content of European maize varieties. *Journal of Economic Entomology*, **97**, 1335–1341.

Urias-Lopez, M. A., Meinke, J. L. (2001): Influence of western corn rootworm (*Coleoptera: Chrysomelidae*) larval injury on yield of different types of maize. *Journal of Economic Entomology,* **94**, 106–111.

Urias-Lopez, M., Meinke, L. J., Higley, L. G., Haile, F. J. (2000): Influence of western corn rootworm (*Coleoptera: Chrysomelidae*) larval injury on photosynthetic rate and vegetative growth on different types of maize. *Environmental Entomology*, **29**, 861–867.

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YIELDING ABILITY OF DIFFERENT TYPES OF MAIZE HYBRIDS

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The aim of the research was to find out whether registered hybrids have the best formula or not. The experimental material consisted of four commercial hybrids: BLASK, GROM, WIARUS and WILGA, and 17 experimental hybrids obtained from the same or related lines. The material was divided into the following groups: 1) SC hybrids: BLASK, and GROM and 7 related hybrids, 2) TC hybrids: WIARUS and 8 related hybrids, 3) TC hybrid WILGA and 2 related hybrids.

In the first group one experimental hybrid yielded the same as BLASK, while four other hybrids yielded as well as GROM. In the second group the experimental hybrids did not differ significantly in yield compared to WIARUS. In the third group WILGA appeared to have the optimal formula.

Key words: maize, inbred lines, yield, types of hybrids

Introduction

Depending on the number of inbred lines used in the hybrid formula, the following types of maize hybrids can be distinguished: single cross (SC) based on the formula A × B; three-way hybrid (TC) with the formula A × B / C and double cross (DC) based on A × B / C × D. In countries with well-developed agriculture it is hard to find double cross hybrids due to their lower yield and worse uniformity compared to SC and TC hybrids (Troyer, 1996).

In Europe, especially in regions with less favourable climates, the seed production of SC hybrids is very expensive and risky, because the inbred lines used as ear parent usually have low yields and might not reach physiological maturity before being killed by autumn frost. For this reason many TC hybrids are widely grown, which exceed SC in terms of yield potential (Heimann and Siodmiak, 2006). Besides SC and TC hybrids, breeders can create modified formulas provided that closely related lines are available.

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The ultimate goal of such modification is improved seed production if it does not have an adverse effect on the yield of the final commercial hybrid; sister line crosses are superior to lines, having better yield and reliability as ear parent and producing more pollen grains and shedding them over a longer period when used as pollinator.

The objectives of this study were to:

– attempt to modify the formula of two SC commercial hybrids, GROM and BLASK, so that the resulting hybrids would equal the performance of the original hybrids, while making seed production much easier.

– find out whether the commercial TC hybrids WIARUS and WILGA had optimal formulas or whether better yielding combinations could be found among the same and sister lines.

Materials and methods

The research material consisted of four commercial hybrids listed on the Polish National List:

- BLASK and GROM – medium early SC hybrids, FAO 240–250
- WIARUS – early TC, FAO 220
- WILGA – very early TC, FAO 180
- 17 experimental hybrids based on lines from the hybrids listed above and lines related to them

All possible crosses were made in 2002. The resulting hybrids were divided into three groups:

First group:

1. BLASK................... S41796 * S41234A-2
2. GROM.................... S41789 * S41324A-2
3. SM 1....................... S41796 * S41336
4. SM 2....................... S41789 * S41336
5. SM 3....................... S41796 / S41336 * S41324A-2
6. SM 4....................... S41789 / S41336 * S41324A-2
7. SM 5....................... S41796 * S41789 / S41324A-2
8. SM 6....................... S41796 * S41789 / S41336
9. SM 7....................... S41796 * S41789 / S41336 * S41324A-2

(S41324A-2 is a sister line to S41336, and S41789 to S41796)

Second group:

1. WIARUS................ S245 * S330 / S41324A-2
2. SM 8....................... S245 * S330 / S41336
3. SM 9....................... S245 * S330 / S41336 * S41324A-2
4. SM 10..................... S245 * S41324A-2
5. SM 11..................... S245 * S41336
6. SM 12..................... S245 / S41336 * S41324A-2
7. SM 13..................... S330 * S41324A-2
8. SM 14..................... S330 / S41336
9. SM 15..................... S330 / S41336 * S41324A-2

Third group:

1. WILGA................... S311 * Co255 / S359
2. SM 16..................... S311 * S359
3. SM 17..................... Co255 * S359

The whole set of 21 hybrids was evaluated in 2003 and 2004 in grain trials in three locations: Smolice, Lagiewniki and Kobierzyce (6 environments), in a randomized complete block design with 3 replications, each 5 m^2 plot consisting of two rows.

Results

Analysis of variance for grain yield, calculated on data collected from the experiment, did not show statistically significant differences in years and locations. For other sources of variation, such as hybrids, environments, genotype-by-location, genotype-by-year and genotype-by-environment, there were significant differences (Table 1).

The analysis of the main effect for the grain yield of hybrids and their interaction with environments is shown in Table 2. Statistically significant differences were found for 8 out of 21 tested hybrids.

Table 1

Analysis of variance for grain yield of tested hybrids, locations and years

Source of variation	Degrees of freedom	Sum of squares	Mean square	F Stat.	F critical values at 0.05
Years	1	177.08	177.08	7.55	18.51
Locations	2	179.87	89.94	3.84	19.00
Environments	2	46.88	23.44	150.16*	3.03
Genotype	20	228.99	11.45	71.56*	1.62
Genotype × year	20	16.15	0.81	5.06*	1.62
Genotype × location	40	16.23	0.41	2.56*	1.45
Genotype × environment	40	12.29	0.31	1.97*	1.45
Regression on explanatory variable	20	7.02	0.35		
Regression deviation	20	5.27	0.26	1.69*	1.62
Experimental error	240		0.16		

*Significant at the LSD (P=0.05) level

Table 2

Estimation of main effects for grain yield of hybrids and their interaction with environment

Hybrid	Formula	Value of main effect	F stat. for main effect	F statistic for interaction with environments
BLASK	S41796*S41324A-2	2.06	42.52*	4.03*
GROM	S41789*S41324A-2	1.28	18.54*	3.58*
SM 1	S41796*S41336	0.12	1.62	0.37
SM 2	S41789*S41336	0.19	1.12	1.31
SM 3	S41796/S41336*S41324A-2	1.34	11.12	6.49*
SM 4	S41789/S41336*S41324A-2	0.88	76.31*	0.41
SM 5	S41796*S41789/S41324A-2	0.83	26.39*	1.06
SM 6	S41796*S41789/S41336	0.02	0.01	1.32
SM 7	S41796*S41789/S41336*S41324A-2	0.71	80.70*	0.25
WIARUS	S245*S330/S41324A-2	0.17	1.11	1.05
SM 8	S245*S330/S41336	-0.40	9.63	0.65
SM 9	S245*S330/S41336*S41324A-2	0.48	5.16	1.8
SM 10	S245*S41324A-2	0.47	4.84	1.85
SM 11	S245*S41336	0.14	2.42	0.34
SM 12	S245/S41336*S41324A-2	0.36	10.04	0.53
SM 13	S330*S41324A-2	0.30	1.24	2.94
SM 14	S330*S41336	-0.23	1.14	1.90
SM 15	S330/S41336*S41324A-2	0.33	1.65	2.60
WILGA	S311*Co255/S359	-2.83	88.83*	3.65*
SM 16	S311*S359	-2.96	80.83*	4.39*
SM 17	Co255*S359	-3.26	519.73*	0.82
Critical values at F 0.05			18.51	3.03

*Significant at the LSD (P=0.05) level

In the first group a significantly higher yield was obtained for the hybrids BLASK, GROM, SM 3, SM 4, SM 5 and SM 7. The hybrids BLASK, GROM and SM 3 did not have stable yields in different environments, as can be concluded from the F statistic for the interaction with environment. In the second group none of the hybrids were significantly different in terms of main effect; moreover, the stable yields of these hybrids show the lack of interaction with environment. All hybrids from the third group had the lowest yields, i.e. the highest significant minus values for the main effect. Hybrid WILGA and SM 16 also showed a significant interaction with environment.

The results of dry matter content in the grain at harvest and the interaction with environment for this trait are shown in Table 3. Out of the 21 hybrids evaluated only five did not have significant differences for the main effect. All hybrids from the first group had minus values, which were significant for all but the hybrid SM 7. Very high values of the F statistic (above 100) and minus values of the main effect were observed for three hybrids: BLASK, GROM and SM 5. BLASK exhibited a significant interaction with environment. It is a full season hybrid (under Polish conditions of grain production) and in favourable years is able to accumulate more dry matter in the grain. By contrast, GROM and SM 5 did not show a significant interaction with environment. They are full season hybrids, with stable performance and a slow accumulation of dry matter in the grain in all environments.

Table 3

Estimation of main effects for dry matter content of hybrids and their interaction with environment

Hybrid	Formula	Value of main effect	F stat. for main effect	F statistic for interaction with environments
BLASK	S41796*S41324A-2	–2.94	109.62*	3.45*
GROM	S41789*S41324A-2	–2.38	113.73*	2.18
SM 1	S41796*S41336	–1.08	8.56*	5.92*
SM 2	S41789*S41336	–1.63	11.84*	9.77*
SM 3	S41796/S41336*S41324A-2	–1.16	11.66*	5.05*
SM 4	S41789/S41336*S41324A-2	–1.58	17.51*	6.25*
SM 5	S41796*S41789/S41324A-2	–2.01	104.11*	1.70
SM 6	S41796*S41789/S41336	–1.08	26.14*	1.94
SM 7	S41796*S41789/S41336*S41324A-2	–0.72	5.82	3.92*
WIARUS	S245*S330/S41324A-2	–0.92	14.78*	2.48*
SM 8	S245*S330/S41336	–0.49	3.32	3.14*
SM 9	S245*S330/S41336*S41324A-2	–0.18	0.56	2.48*
SM 10	S245*S41324A-2	–1.03	14.75*	3.13*
SM 11	S245*S41336	–0.73	10.87*	2.16
SM 12	S245/S41336*S41324A-2	0.66	2.26	8.46*
SM 13	S330*S41324A-2	–0.97	15.62*	2.64*
SM 14	S330*S41336	–0.99	11.83*	3.61*
SM 15	S330/S41336*S41324A-2	0.09	0.38	1.03
WILGA	S311*Co255/S359	6.21	85.12*	19.76*
SM 16	S311*S359	6.45	123.87*	14.67*
SM 17	Co255*S359	6.48	213.05*	8.62*
Critical values at F 0.05			6.03	2.30

*Significant at the LSD (P=0.05) level

In the second group only SM 12 and SM 15 had positive values, although these were not statistically significant. The remaining hybrids had minus values, which were statistically significant for five of them (WIARUS, SM 10, SM 11, SM 13, SM 14). All the hybrids from the second group, with the exception of SM 11 and SM 15, showed a significant interaction with environment, so stability in dry matter accumulation is not their strong point: environmental conditions affect them greatly.

All hybrids from the third group had significantly positive values for the main effect. They are very early-maturing hybrids, accumulating dry matter rapidly, but they are not very stable and are easily affected by environmental conditions (high significant values of the F statistic for interaction).

Discussion

The formula of the hybrid BLASK was found to be optimal; the yield of 12.68 t/ha was significantly higher than for any other hybrid from the first group with the exception of SM 3 (Table 4). Hybrid SM 3, with the formula S41796 / S41336 × S41324A-2 is interesting from two points of view: firstly, a grain yield similar to that of BLASK was harvested at 1.8% higher dry matter content, secondly, the single cross used as pollinator would allow the planting of more rows with ear parent (8:2) in seed production, as opposed to the typical 4:2 ratio.

Yields similar to that of GROM (11.90 t/ha) were produced by four other hybrids with similar formulas: SM 3, SM 4, SM 5 and SM 7. Two hybrids, SM 3 and SM 7, also had significantly higher dry matter content in the grain. From the seed production point of view the most interesting are hybrids SM 5 and SM 7 because of their more productive ear parent. Their grain yield stability over the environments was also good. Another interesting hybrid was SM 4, with the formula S41789 / S41336 × S41324A-2, the grain yield and dry matter content of which did not differ significantly from that of GROM. Again, the seed production of this hybrid would be much more profitable, since the single cross S41336 × S41324A-2 can provide enough pollen grains for eight rows of ear parent instead of four (in the case of GROM seed production).

There were no significant differences in yield and dry matter content among the hybrids from the second group, based on lines S245, S330 and S41324A-2. Single crosses made in this group did not exhibit superiority in yield compared to other hybrids, so the formula of the registered TC hybrid WIARUS is optimal (Table 4). One interesting hybrid seems to be SM 9, with a modified three-way cross formula (MTC): S245 × S330 / S41336 × S41324A-2, the yield and dry matter content of which did not differ significantly from WIARUS. Hybrid SM 9 proved its stability in grain yield over all environments. In seed production the single cross used as a pollinator would provide enough pollen grains for eight rows of ear parent instead of four (in the case of WIARUS seed production). A comparison of the modified single cross SM 15 (formula

S330/S41336 × S41324A-2) with the SC hybrids SM 13 and SM 14 (formulas S330 × S41324A-2 and S330 × S41336 respectively), showed significantly higher dry matter content in the grain of SM 15 (1.06% and 1.08%, respectively) while equalling the grain yield level of the SC hybrids. Also, the seed production of hybrids, SM 12 and SM 15, with modified formulas, would be much cheaper.

The lowest yields were observed in the third group of hybrids, based on the lines used in the formula of the hybrid WILGA (S311, Co255 and S359). At the same time, they showed the highest dry matter content in the grain at harvest. Two possible SC hybrids, SM 16 and SM 17 (formulas S311 × S359 and Co255 × S359, respectively) did not differ significantly for yield and dry matter content compared to the TC hybrid WILGA. One distinguishing feature of SM 17 is its better yield stability.

Table 4

Mean values (2003–2004) and deviation from group mean for grain yield (t/ha) and dry matter content (%) in the grain at harvest

No.	Hybrid	Formula	Grain yield		Dry matter content	
			Mean	Deviation	Mean	Deviation
1	BLASK	S41796×S41324A-2	12.68	+1.23	71.19	–1.42
2	GROM	S41789×S41324A-2	11.90	+0.45	71.56	–1.05
3	SM 1	S41796×S41336	10.74	–0.71	73.06	+0.55
4	SM 2	S41789×S41336	10.81	–0.63	73.51	+1.10
5	SM 3	S41796/S41336×S41324A-2	11.96	+0.51	72.98	+0.38
6	SM 4	S41789/S41336×S41324A-2	11.50	+0.05	72.56	–0.05
7	SM 5	S41796×S41789/S41324A-2	11.45	0.00	72.13	–0.48
8	SM 6	S41796×S41789/S41336	10.64	–0.81	73.06	+0.55
9	SM 7	S41796×S41789/S41336×S41324A-2	11.33	–0.12	73.42	+0.81
		Mean of group I	11.45		72.61	
10	WIARUS	S245×S330/S41324A-2	10.79	–0.01	73.22	–0.33
11	SM 8	S245×S330/S41336	10.23	–0.57	73.65	+0.10
12	SM 9	S245×S330/S41336×S41324A-2	11.10	+0.30	73.96	+0.41
13	SM 10	S245×S41324A-2	11.09	+0.29	73.11	–0.44
14	SM 11	S245×S41336	10.76	–0.04	73.41	–0.14
15	SM 12	S245/S41336×S41324A-2	10.98	+0.19	74.08	+0.53
16	SM 13	S330×S41324A-2	10.92	+0.12	73.17	–0.38
17	SM 14	S330×S41336	10.39	–0.41	73.15	–0.40
18	SM 15	S330/S41336×S41324A-2	10.95	+0.15	74.23	+0.68
		Mean of group II	10.80		73.55	
19	WILGA	S311×Co255/S359	7.79	+0.19	80.34	–0.18
20	SM 16	S311×S359	7.66	+0.06	80.59	+0.07
21	SM 17	Co255×S359	7.36	–0.24	80.62	+0.10
		Mean of group III	7.60		80.52	
Grand mean			10.62		74.14	
LSD (P=0.05)			0.77		1.01	

Conclusions

In the first group of hybrids, based on formulas similar to those of the registered SC hybrids BLASK and GROM, BLASK proved to be definitely the best yielder, but also the latest. Another hybrid, SM 3 (formula S41796 / S41336 × S41324A-2) had a yield similar to that of BLASK, but had significantly higher dry matter content in the grain and would make seed production easier.

The experimental hybrids SM 4, SM 5 and SM 7 yielded at the same level as GROM. Especially SM 4 and SM 7, hybrids with significantly higher dry matter content, are worth noting due to their easier seed production compared with the SC hybrid GROM.

There were no significant differences in yield and dry matter content in the second group of hybrids, based on formulas similar to those of the registered TC hybrid WIARUS. Among the eight experimental hybrids, SM 9 is worth noting due to its easier seed production.

In the third group of hybrids, based on the lines used in the formula of the registered TC hybrid WILGA, no significant differences in grain yield or dry matter content were observed between WILGA and the two experimental SC hybrids.

References

Heimann, H., Siodmiak, J. (2006): Syntezy winikow doswiadczen rejestrowych. Kukurydza 2005. (Summary of results of official trials. Maize 2005.) *Zeszyt*, **46**, COBORU Slupia Wielka, 21. p.

Troyer, A. F. (1996): Breeding widely adapted popular maize hybrids. *Euphytica,* **92**, 163–174.

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EFFECT OF CROP PRODUCTION FACTORS ON THE YIELD AND YIELD STABILITY OF MAIZE (*Zea mays* L.) HYBRIDS

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The effect of sowing date, N fertiliser rate, plant density and genotype on the yield stability of maize was analysed using 15-year data from a 5×4×5-factorial sowing date experiment, 35-year data from a two-factorial N fertilisation experiment and 25-year data from a two-factorial plant density experiment. Stability analysis on the experimental treatments was carried out using the variance and regression methods. Among the variance parameters, the ecovalence (W), the stability variance (σ^2) and the yield stability (YS) were calculated.

Based on the data of the sowing date experiment the optimum sowing date (Apr. 24) or sowing ten days later (May 5) were found to be the most stable due to the low, non-significant values of the variance parameters and the values close to unity for the regression coefficients (b). Although early sowing (Apr. 14) led to a significantly higher yield than late sowing, the yield stability was poorer for early sowing. In the long-term N fertilisation experiment the variance parameters indicated the least yield fluctuation at N rates of 80 and 160 kg ha^{-1}, though the yield stability (YS) parameter for the 240 kg ha^{-1} N rate was also above-average. Regression analysis showed that the yield level and yield stability were the same in all environments for the 160 and 240 kg ha^{-1} N rates. The stability of the 80 kg ha^{-1} N rate was similar, but the yield level was approx. 1.3 t ha^{-1} lower. The yield stability of the plant density response of the maize hybrids was different in each maturity group (FAO number). The stable plant density range was broadest (50–90 thousand plants ha^{-1}) in the FAO 200–299 group. As the vegetation period lengthened the stable plant density range narrowed and shifted towards lower plant densities (for the FAO 400–499 and FAO 500–599 maturity groups: 50–70 thousand plants ha^{-1}).

The variance and regression parameters of stability analysis both contributed to the characterisation of the stability of the genotypes and cropping systems investigated. It can be concluded from the results that high yields and yield stability are not necessarily mutually exclusive.

Key words: maize, sowing date, N fertilisation, plant density, variance and regression methods

Introduction

The genotype × environment interaction is a major concern in yield trials for two main reasons. First it reduces progress from selection, and second, it makes cultivar (or cropping system) recommendation difficult because it is statistically impossible to interpret the main effects. Agricultural scientists must seek effective statistical tools to mitigate the challenges caused by interaction (Kang and Gauch, 1996).

Stability analysis is often used to interpret the significant treatment × environment interactions observed in the models used for analysis of variance in long-term trials and experimental series. The notion of stability implies that there is a random, unpredictable element in the performance of a cropping system. The larger this random component, the smaller the stability of a system. The mean (fixed, i.e. systematic effect) and the variance (random element) are the two main parameters describing the response pattern of a cropping system (Piepho, 1998). Different approaches to stability analysis differ in how the random term is further partitioned.

In agricultural research the analysis of yield stability has been largely confined to multi-environmental trials on crop cultivars. The idea of applying methods of stability analysis to cropping systems is not novel (Willey, 1979; Mead and Riley, 1981). Hildebrand (1984), Raun et al. (1993) and Guertal et al. (1994) employed stability analysis in long-term experiments to evaluate fertilisation treatments. Piepho (1998) emphasized that methods for comparing the stability of cultivars could also be used for comparing different agronomic treatments in general, of which cultivars are only a special case.

The measurement of yield stability over time involves at least three components: (1) the relationship of yield with the local environment, (2) the average yield level and (3) the variability of the yield (Mead et al., 1986). Univariate stability measures are divided into two broad categories that use either variance or regression methods. The most important variance parameters are the within-treatment mean square (s^2), the coefficient of variation (CV%), the ecovalence (W) (Wricke, 1962) and the stability variance (σ^2) (Shukla, 1972). The two latter parameters measure the contribution of the treatment to the treatment × environment interaction (Callaway and Francis, 1993). There are several approaches that combine the mean and variance of a system. Recently Kang (1993) developed a yield stability (YS) statistic that combines yield and stability of performance into a single selection criterion.

A common approach to stability analysis is to regress the performance of the system onto an environmental index computed as the mean of all observations in an environment. This index may be taken as a measure of the productivity of the environment. The regression techniques used to develop stability parameters are based on linear slope and deviation from that slope. Systems where the regression has a relatively large slope show an above-average response to improved environmental index. The regression approach was first suggested by Yates and Cochran (1938), followed later by Finlay and Wilkinson (1963) and Eberhart and Russell (1966). A stable system has been defined as the one that changes least in response to changes in the environment. Finlay and Wilkinson (1963) suggested that slopes with $b < 1.0$ indicated better adaptation to poor environments, while genotypes with $b > 1.0$ are best used in superior environments. A cropping system with an estimate of b equal to unity shows an average response to environmental conditions, as measured by the environmental mean.

The aim of the research was: (a) to evaluate the effects of various planting dates, N fertilisation rates and plant densities on the yield of maize hybrids with different vegetation periods, and (b) to use conventional analysis of variance as well as the variance and regression methods of stability analysis to characterise the effect of experimental treatments on yield stability.

Materials and methods

Experiments and experimental treatments

Sowing date experiment

This experiment was originally set up in the institute nursery by Béla Győrffy and his colleagues in 1980 as a nitrogen fertilisation experiment, in a block design with four replications. The N treatments were: 0, 60, 120, 180 and 240 kg ha^{-1}. In all the treatments the P and K fertiliser rates were the same (120 kg ha^{-1}). In 1991 the experiment was converted into a 5×4×5-factorial sowing date experiment in a split-split-plot design, with the same N treatments in the main plots, sowing dates in the subplots and maize hybrids in the sub-subplots. The four sowing date treatments involved sowing at the optimum date (around Apr. 24), or sowing 10 days before (early), 10 days after (late) or 20 days after this date (very late). The five maize hybrids (H_1–H_5) were chosen to represent different maturity groups (H_1: FAO 200–299, H_2, H_3: FAO 300–399, and H_4, H_5: FAO 400–499). The size of the main plot was 30 × 6 m and that of the subplots 7.5 × 6 m, while the hybrids forming the sub-subplots were each sown in two rows, separated by buffer rows. The soil of the experimental area was a humous loam of the chernozem type with forest residues, mildly alkaline in the ploughed layer, with a humus content of 3.3–3.6% and good supplies of phosphorus and potassium.

Long-term N fertilisation experiment

The effect of N fertiliser on the N fertiliser response of the maize hybrids was examined in a long-term small-plot experiment set up by Béla Győrffy and colleagues in 1961. The N fertiliser treatments were as follows: 0, 80, 160 and 240 kg ha^{-1}. The P and K fertiliser rates were the same in all the treatments (160 kg ha^{-1}). The experiment was set up in a random block design with four replications, with N treatments in the main plots (193 m^2) and maize hybrids in the subplots (6.4 m^2). Since 1970 the N fertiliser response of 10–12 maize hybrids has been investigated in a maize monoculture each year. The soil of the experimental area was a humous loam of the chernozem type with forest residues, mildly acidic in the ploughed layer, with poor supplies of available phosphorus and good supplies of potassium. The area had a poor water regime and was partially eroded due to its hilly location.

Plant density experiment

The effect of plant density on the grain yield of maize was examined at seven plant densities in the 30–90 thousand plants ha^{-1} range (with differences of 10^4 between treatments) on chernozem soil with forest residues in the institute nursery. The small-plot experiment was set up in a split-plot design (main plot: plant density, subplot: hybrid) in four replications. Each year the plant density responses of 30–45 maize hybrids were investigated up till 1990, after which the number was reduced to 18–20 hybrids. In all treatments the row distance was the same (70 cm), while the plant distance ranged from 14 to 71 cm, depending on the treatment. Each year the plots were fertilised with 200 kg N, 160 kg P_2O_5 and 160 kg K_2O per hectare.

Variance and regression methods of stability analysis

The stability analysis of the experimental treatments was carried out using the variance and regression methods. Among the variance parameters, Wricke's (1962) ecovalence (W) and Shukla's (1972) stability variance (σ^2) parameter were calculated, while Kang's (1993) yield stability (YS) parameter was calculated using the STABLE model of Kang and Magari (1995). This program allows covariants to be used to eliminate the heterogeneity (non-additive or linear effect of the covariant) from the treatment × environment interaction. In the stability analysis the rainfall sum (mm) during the vegetation period (Apr.–Sep.) was the covariant. The data required to run the program are the treatments (genotypes, cropping systems), the environment (location, year) and the number of replications, together with the pooled experimental error calculated from annual analysis of variance (ANOVA). The stability analysis program first generates the ANOVA results table with the genotype (cropping system), environment and interaction components, and the breakdown of the interaction into heterogeneity and residual components. It then indicates the contribution of each treatment to the interaction, by calculating the stability variance (σ^2) and ecovalence (W) parameters, the $LSD_{5\%}$ value for the yield response and the yield stability (YS) parameter, and selects the treatments where the YS value is greater than average.

In the regression method of stability analysis the regression between the experimental treatment and the environmental index is calculated. The environmental index is the mean of each treatment in the given environment (year), expressing the productivity of the given location. Linear regression analysis was carried out according to the methods of Finlay and Wilkinson (1963) and Raun et al. (1993). The effect of the treatments was characterised not only by the steepness of the straight lines, but also from the intersections of the linear functions under diverse environmental conditions, as proposed by Raun et al. (1993). The data were processed using the MSTAT-C and SPSS 14.0 programs.

Results

Results of analysis of variance

Table 1 presents the ANOVA results for the stability analysis of each experiment, including the degrees of freedom (d.f.), the mean squares (MS) and the significance of the F values. As can be seen from the table, the interaction was significant in all cases. A comparison of the MS values for treatments (cropping systems) and environment indicates that in the sowing date experiment the effect of N fertilisation surpassed that of the environment, while the sowing date and the genotype had less effect than the environment. In the long-term N fertilisation experiment the long-term effect of N fertiliser was dominant, with an MS value almost 28 times that of the environment MS. In the plant density experiment the environment MS value was more than six times that of the plant density MS.

Table 1

Combined table for analysis of variance, based on the stability analysis program of Kang (1993)

Source	Factorial sowing date experiment						Long-term exp.		Experimental series	
	N fertiliser		Sowing date		Genotype		N fertilisation		Plant density	
	d.f.	M.S.	d.f.	M.S.	d.f.	M.S.	d.f.	M.S.	d.f.	M.S.
Cropping systems	4	58.22^{***}	3	15.19^{***}	4	29.05^{***}	3	689.85^{***}	6	20.94^{***}
Environments	14	43.70^{***}	14	34.96^{***}	14	43.70^{***}	34	24.89^{***}	24	129.40^{***}
Interaction	56	2.06^{***}	42	1.59^{***}	56	1.78^{***}	102	1.64^{***}	144	1.15^{***}
Heterogeneity	4	0.48^{NS}	3	0.12^{NS}	4	0.09^{NS}	3	0.13^{NS}	6	0.53^{NS}
Residual	52	2.19^{***}	39	1.70^{***}	52	1.91^{***}	99	1.69^{***}	138	3.70^{***}
Pooled error	180	0.33	135	0.33	180	0.33	315	1.19	450	0.32

***Significant at the P = 0.1% level, ^{NS} = non-significant

Effect of sowing date, N fertilisation and maize hybrid on yield stability (1991–2005)

The effect of sowing date, N fertilisation and maize hybrid on yield stability was characterised using the mean yield response, based on the 1991–2005 data of the three-factor experiment, and on the variance and regression parameters (Table 2). A comparison of the various sowing dates showed that the value of the coefficient of variance (CV%) was greatest in the very late and early treatments, and smallest when the experiment was sown at the optimum date or ten days later. Among the N fertiliser treatments the CV% had the highest value in the control plot with no N fertilisation and the lowest value in the 60–180 kg ha^{-1} N range. The values of ecovalence (W) and stability variance (σ^2) were lowest and non-significant for the optimum sowing date and ten days later. On the basis of mean yield response and stability, Kang's (1993) yield stability (YS) parameter indicated that sowing at the optimum date (Apr. 24) or ten days later was the most favourable.

The control treatment without N fertilisation and the excessively high (240 kg ha^{-1}) N rate were responsible for the significant N × year interaction. The W and σ^2 parameters were not significant at N rates of 60, 120 and 180 kg ha^{-1}. In the same way, the YS parameter found the 60, 120 and 180 kg ha^{-1} rates of N fertiliser to be the most efficient. There was no difference between the stability variance (σ^2) and ecovalence (W) parameters of the hybrids investigated; all the hybrids made a significant contribution to the genotype × environment interaction. The yield stability (YS) parameter, however, which considers both the yield and its stability, indicated that hybrids in FAO maturity groups 300–399 and 400–499 (H_3–H_5) were the most favourable (Table 2).

The effect of sowing date, N fertilisation and maize hybrid on yield stability is illustrated in Figure 1 on the basis of regression analysis. A comparison of the various sowing dates revealed regression coefficients (b) with values greater than 1.0 for the optimum sowing date and ten days later (1.045 and 1.076, respectively). In all the environments the late and very late sowing dates led to lower yields than early sowing. The regression coefficients indicated that the 120 kg ha^{-1} N rate had the greatest stability (b = 1.065) of all the N fertiliser treatments. In an unfavourable environment (environmental mean < 4 t ha^{-1}) the yield was greater at a lower fertilisation level (60 kg ha^{-1} N), while in a favourable environment N rates of 120 kg ha^{-1} or more had a substantial yield-increasing effect. The b = 0.56 value registered for the control treatment is indicative of adaptation to the unfavourable environment. Regression analysis revealed that hybrids belonging to the FAO maturity groups 400–499 and 300–399 (H_5, H_4) had greater yield and yield stability in all the environments. The hybrid in the FAO 200–299 group (H_1) had the lowest yield in all the environments (Fig. 1).

Effect of N fertilisation on yield stability in a long-term maize monoculture (1970–2005)

The effect of N fertilisation on the yield stability of maize hybrids was investigated on the basis of 35 years of data from a long-term experiment on maize grown in a monoculture. The effect of N fertilisation on the variance and regression parameters can be seen from the data in Table 3. Among the variance parameters, CV%, W and σ^2 had the lowest values at N rates of 80 and 160 kg ha^{-1}. The non-significant values obtained for W and σ^2 were an indication that these treatments did not contribute to the significant treatment × environment interaction.

Table 2

Effect of sowing date, N fertilisation (kg ha^{-1}) and maize hybrid on the yield (t ha^{-1}) and yield stability in a three-factorial experiment (1991–2005)

Treatments	Yield response	Variance parameters				Regression parameters[‡]		
		CV%	W	σ^2	YS[§]	r	b	a
Sowing date								
Apr. 15	8.59 a	7.27	20.37	$2{,}12^{***}$		0.923^{***}	0.977	0.551
Apr. 24	8.58 a	4.07	6.58	0.25^{NS}	+	0.977^{***}	1.045	–0.023
May 5	8.24 b	4.59	8.16	0.37^{NS}	+	0.950^{***}	1.076	–0.619
May 16	7.52 c	10.16	31.48	3.70^{**}		0.876^{***}	0.902	0.091
N fertiliser								
0	6.50 c	13.33	68.81	7.51^{***}		0.668^{**}	0.506	2.328
60	8.53 b	2.82	3.62	0.26^{NS}	+	0.986^{***}	0.931	0.865
120	8.87 a	3.21	4.76	0.12^{NS}	+	0.985^{***}	1.065	0.101
180	8.81 a	4.01	10.01	0.50^{NS}	+	0.981^{***}	1.170	–0.821
240	8.45 b	6.42	28.41	2.69^{**}		0.966^{***}	1.327	–2.473
Maize hybrid								
H_1	7.25 a	9.27	24.10	2.28^{***}		0.905^{***}	0.931	–0.409
H_2	8.05 b	6.66	15.70	1.28^{***}		0.935^{***}	0.923	0.460
H_3	8.34 c	7.73	21.70	1.99^{***}	+	0.918^{***}	0.974	0.320
H_4	8.31 c	5.41	11.47	0.77^{**}	+	0.966^{***}	1.090	–0.658
H_5	9.19 d	7.66	26.63	2.58^{***}	+	0.921^{***}	1.082	0.286

CV: coefficient of variance; W: ecovalence; σ^2: stability variance; YS: yield stability; r: correlation coefficient; b: regression coefficient; a: regression constant. Within each treatment, yields designated with the same letter do not differ significantly from each other according to Duncan's Multiple Range Test. ***, ** Significant at the P = 0.1% and P = 1% levels, respectively; ^{NS} = non-significant. [§]: + indicates the best treatments on the basis of yield stability; [‡]: Number of data pairs (years) in the regression analysis: n = 15.

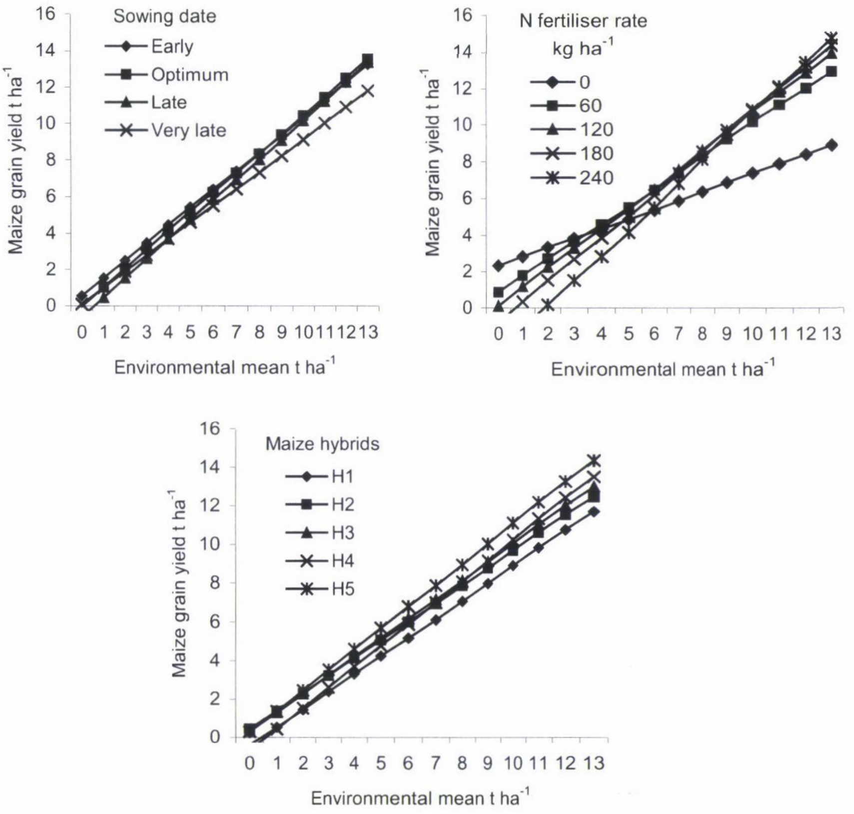

Fig. 1. Effect of sowing date, N fertilisation and maturity group on the yield stability of maize (1991–2005)

Table 3

Effect of N fertilisation (kg ha^{-1}) on the yield (t ha^{-1}) and yield stability of maize hybrids grown in a monoculture in a long-term experiment (1970–2005)

N fertiliser rate	Yield response	Variance parameters				Regression parameters		
		CV%	W	σ^2	YS§	r	b	a
0	3.701 c	14.19	84.98	4.18^{***}		0.618^{**}	0.727	–1.312
80	7.055 b	1.48	15.34	0.08^{NS}	+	0.974^{***}	1.086	–0.429
160	8.398 a	1.94	22.98	0.53^{NS}	+	0.959^{***}	1.084	0.930
240	8.410 a	3.79	44.40	1.79^{***}	+	0.927^{***}	1.103	0.811

For the names of variance and regression parameters, see Table 2. Number of data pairs (years) in the regression analysis: n = 35.

The effect of various N fertiliser treatments on maize yield stability is illustrated in Figure 2, based on the linear regression model. The regression coefficient was smallest (b = 0.727) in the control treatment without N fertilisation, while in the remaining treatments it ranged from 1.084 to 1.103. The value closest to unity was obtained for the 160 kg ha^{-1} treatment (1.084), which had the best stability and yield level in the environment in question (Table 3). The positive value of the regression constant (a) in the N_{160} and N_{240} treatments was indicative of the higher yield level in these treatments compared with the negative values obtained for the N_0 and N_{80} rates. An analysis of the homogeneity of the regression coefficients indicated a significant difference between the values obtained for the N_0 and N_{80} treatments (t-value: 3.58***, n = 35). Consequently, the yield stability of maize was smaller in the control plot than when N fertiliser was applied. The regression method revealed that the yield stability was greatest at a rate of 160 kg ha^{-1} N.

Effect of plant density on yield stability in the experimental series (1981–2005)

The effect of plant density on the variance and regression parameters of the yield stability of maize hybrids is illustrated in Table 4. It can be seen that in the plant density range 50–70 thousand plants ha^{-1} the stability variance (σ^2) parameter was non-significant and the value of the ecovalence (W) parameter was the lowest, indicating that these treatments did not contribute to the significant treatment × environment interaction and that the yield stability was greatest in this plant density range. Kang's (1993) yield stability parameter (which gives sufficient weight to the yield) selected the 50–80 thousand plants ha^{-1} density range. Judging by the value of the regression coefficient (b), a plant density of 60,000 plants ha^{-1} gave the greatest stability (Table 4). The plotting of linear functions (Fig. 3) revealed that a plant density of 30–50 thousand plants ha^{-1} had greater stability when the environmental mean was below 5 t ha^{-1}, while above this level plant densities of 60,000 plants ha^{-1} or more had greater yield stability.

Table 4

Effect of plant density (1000 plants ha^{-1}) on the yield (t ha^{-1}) and on the variance and regression parameters of the yield stability of maize hybrids (1981–2005)

Plant density	Yield response	Variance parameters			Regression parameters		
		W	σ^2	YS§	r	b	a
30	6.806	62.3	3.40***		0.934	0.670	1.636
40	7.389	20.3	0.95***		0.990	0.823	1.045
50	7.827	9.5	0.33^{NS}	+	0.991	0.928	0.671
60	8.070	4.4	0.03^{NS}	+	0.997	1.059	–0.095
70	7.975	10.0	0.35^{NS}	+	0.998	1.130	–0.741
80	7.990	21.4	1.02***	+	0.993	1.169	–1.025
90	7.919	38.0	1.98***		0.988	1.220	–1.490
$LSD_{5\%}$	0.131						

For the names of variance and regression parameters, see Table 2. Number of data pairs (years) in the regression analysis: n = 25.

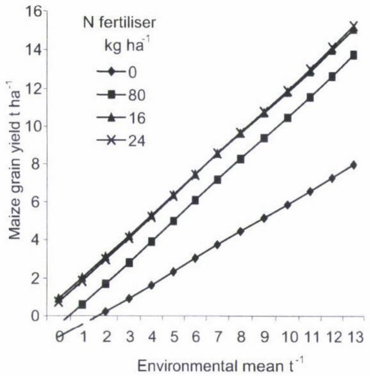

Fig. 2. Effect of N fertilisation on the yield stability of maize hybrids in a long-term monoculture experiment (1970–2005)

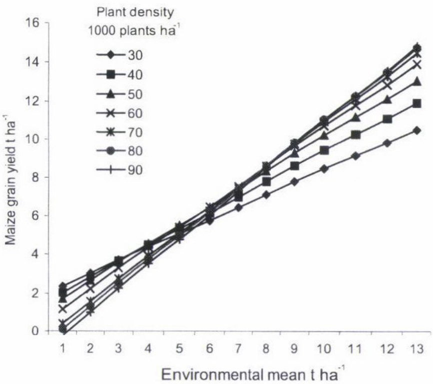

Fig. 3. Effect of plant density on the yield stability of maize hybrids between 1981 and 2005 (averaged over 20–45 maize hybrids each year)

In the next step of the analysis the plant density-dependent yield stability of the maize hybrids was compared for various maturity groups. The plant density-dependent variance parameters for each FAO number are given in Table 5, from which it is clear that the plant density-dependent yield stability of the maize hybrids was different for each maturity group. The significant values of σ^2 for the 30–40 and 80–90 thousand plants ha^{-1} plant densities in all the maturity groups indicated the poorer stability of these treatments. In the FAO 200 and FAO 400 groups the non-significant σ^2 values obtained for plant densities of 50, 60 and 70 thousand ha^{-1} suggest that these treatments were more stable. The stability variance parameter was non-significant for plant densities of 60 and 70 thousand plants ha^{-1} in the FAO 300 maturity group and 50 and 60 thousand ha^{-1} in the FAO 500 group. According to Kang's (1993) yield stability parameter (YS) the most stable plant density treatments were 50–90 thousand plants ha^{-1} in the FAO 200 group, 70–90 thousand plants ha^{-1} in the FAO 300 group, and 50–70 thousand plants ha^{-1} in the FAO 400 and 500 groups. The effect of plant density and maturity group on the yield responses and yield stability regression parameters of the maize hybrids is demonstrated in Table 6. It can be seen from the table that the dependence of the regression coefficients (b) on plant density and maturity group, and the yield responses of the hybrids reveal the same patterns as described above for the variance parameters.

Discussion

Yield stability, which involves the average yield level, the yield variability and the correlation of the yield with the local environment, is an important parameter for the sustainability of crop production. The analysis of several decades of data from various two- and three-factorial crop production experiments revealed the importance of the environment (year) effect and the treatment × environment interaction for the investigation of treatment effects.

Table 5

Effect of plant density (10^3 plants ha^{-1}) and maturity group on the variance parameters of the yield stability of maize hybrids (1981–2005)

Plant density	FAO 200–299			FAO 300–399			FAO 400–499			FAO 500–599		
	σ^2	W	YS	σ^2	W	YS	σ^2	W	YS	σ^2	W	YS
30	2.78^{**}	49.0		3.45^{***}	63.8		3.97^{**}	72.5		5.14^{***}	93.9	
40	0.67^{**}	14.4		1.14^{***}	24.2		1.00^{**}	21.5		1.49^{***}	31.4	
50	0.40^{NS}	9.9	+	0.57^{***}	14.5		0.33^{NS}	10.1	+	0.30^{NS}	10.9	+
60	0.35^{NS}	9.0	+	0.14^{NS}	7.1	+	0.18^{NS}	7.5	+	0.30^{NS}	10.9	+
70	0.29^{NS}	8.1	+	0.37^{NS}	11.1	+	0.43^{NS}	11.8	+	0.83^{***}	20.0	+
80	0.83^{**}	17.1	+	1.38^{***}	28.4	+	1.09^{**}	23.0		0.98^{***}	22.6	
90	1.83^{**}	33.5	+	2.48^{***}	47.2		1.97^{**}	38.1		2.84^{***}	54.6	

For the names of variance and regression parameters, see Table 2

Table 6

Effect of plant density (10^3 plants ha^{-1}) and maturity group on the yield responses (t ha^{-1}) and yield stability regression parameters of maize hybrids (1981–2005)

Plant density	Mean yield response				Regression coefficient (b)				Regression constant (a)			
	1	2	3	4	1	2	3	4	1	2	3	4
30	6.264	6.782	6.942	7.268	0.598	0.584	0.736	0.760	1.658	2.287	1.272	1.414
40	6.825	7.418	7.477	7.802	0.731	0.734	0.871	0.965	1.191	1.767	0.767	0.368
50	7.271	7.896	7.893	8.208	0.814	0.827	0.977	1.131	1.002	1.528	0.367	–0.505
60	7.598	8.074	8.143	8.421	0.922	0.949	1.129	1.266	0.496	0.764	–0.552	–1.333
70	7.647	7.993	8.034	8.188	0.949	1.049	1.182	1.326	0.340	–0.088	–1.071	–2.026
80	7.775	8.067	7.976	8.114	0.989	1.111	1.225	1.324	0.154	–0.491	–1.460	–2.078
90	7.771	7.963	7.858	8.009	1.037	1.168	1.270	1.376	–0.217	–1.031	–1.927	–2.589
$LSD_{5\%}$		0.131										

1: FAO 200–299; 2: FAO 300–399; 3: FAO 400–499; 4: FAO 500–599; Number of data pairs (years) in the regression analysis: n = 25.

In the sowing date experiment the optimum sowing date and sowing ten days later were found to be the most stable, since the variance parameters had low, non-significant values and the regression coefficients (b) were close to unity. These two sowing dates had above-average yield stability (YS) parameters. Although the yield in the early sowing treatment was significantly higher than after late sowing (May 5), early sowing had lower yield stability. Maize had the best yield stability when grown with N rates of 60, 120 or 180 kg ha^{-1}, as indicated by both the variance and regression parameters. At an environmental mean of below 4 t ha^{-1}, however, only N rates of 60 and 120 kg ha^{-1} proved to be stable. In the sowing date experiment maize hybrids with longer vegetation periods had greater yield stability, especially in a favourable environment.

In the long-term N fertilisation experiment the variance parameters indicated the least yield fluctuation in the 80 and 160 kg ha^{-1} N treatments, though the value of the yield stability (YS) index was also above-average for the 240 kg ha^{-1} N rate. Regression analysis demonstrated that the yield stability for rates of 160 and 240 kg ha^{-1} N was the same in all environments. The stability was similar for the 80 kg ha^{-1} N rate, but the yield level was around 1.3 t ha^{-1} lower.

The 25 years of data in the plant density experiment indicated that the most stable plant density range was 50–70 thousand plants ha^{-1}. It could be seen from regression analysis that for an environment mean of below 5 t ha^{-1} a plant density of 30–50 thousand plants ha^{-1} gave greater stability. The yield stability of the plant density response differed for maize hybrids from different maturity groups. The stable plant density range was broadest (50–90 thousand plants ha^{-1}) in the FAO 200–299 group. As the vegetation period lengthened the stable plant density range narrowed and shifted to lower plant densities (50–70 thousand plants ha^{-1} for the FAO 400–499 and 500–599 groups). As reported by Tokatlidis and Koutroubas (2004), the choice of hybrids with a broad optimum plant density range, where the yield maximum is less dependent on high plant density, could make a considerable contribution to an improvement in yield stability.

Yield stability depends on yield components and other plant traits, such as pest resistance, stress tolerance and environmental variables. By identifying and regulating the most limiting factors, stability can be substantially improved (Kang and Gauch, 1996). Under the given experimental conditions, the environmental variable with the greatest influence on yield stability was the rainfall sum during the growing period. All the agronomic factors tested (sowing date, N fertiliser rate, plant density and genotype) had a great influence on yield stability. It is thus important to identify the treatments with the best yield stability for recommendation to farmers. Both the variance and regression parameters of stability analysis were able to give an indication of the stability of genotypes and cropping systems. In agreement with Tollenaar and Wu (1999) it could be concluded from the results that high yields and yield stability are not mutually exclusive.

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References

Callaway, M. B., Francis, C. A. (1993): *Crop Improvement for Sustainable Agriculture*. University of Nebraska Press, Lincoln and London.

Eberhart, S. A., Russell, W. A. (1966): Stability parameters for comparing varieties. *Crop Sci.*, **6**, 36–40.

Finlay, K. W., Wilkinson, G. N. (1963): The analysis of adaptation in a plant breeding programme. *Aust. J. Agric. Res.*, **14**, 742–754.

Guertal, E. A., Raun, W. R., Westerman, R. L., Boman, R. K. (1994): Application of stability analysis for single site, long-term experiments. *Agron. J.*, **86**, 1016–1019.

Hildebrand, P. E. (1984): Modified stability analysis of farmer managed, on-farm trials. *Agron. J.*, **76**, 271–274.

Kang, M. S. (1993): Simultaneous selection for yield and stability in crop performance trials: consequences for growers. *Agron. J.*, **85**, 754–757.

Kang, M. S., Magari, R. (1995): STABLE: A basic program for calculating stability and yield-stability statistics. *Agron. J.*, **87**, 276–277.

Kang, M. S., Gauch, H. G. (1996): *Genotype-by-Environment Interaction*. CRC Press, Boca Raton, New York.

Mead, R., Riley, J. (1981): A review of statistical ideas relevant to intercropping research. *J. Royal Statist. Soc. A*, **144**, 462–509.

Mead, R., Riley, J., Dear, K., Singh, S. P. (1986): Stability comparison of intercropping and monocropping systems. *Biometrics*, **42**, 253–266.

Piepho, H. P. (1998): Methods for comparing the yield stability of cropping systems – a review. *J. Agron. Crop Sci.*, **180**, 193–213.

Raun, W. R., Barreto, H. J., Westerman, R. L. (1993): Use of stability analysis for long-term soil fertility experiments. *Agron. J.*, **85**, 159–167.

Shukla, G. K. (1972): Some statistical aspects of partitioning genotype-environmental components of variability. *Heredity*, **29**, 237–245.

Tokatlidis, I. S., Koutroubas, S. D. (2004): A review of maize hybrids dependence on high plant populations and its implications for crop yield stability. *Field Crops Res.*, **88**, 103–114.

Tollenaar, T., Wu, J. (1999): Yield improvement in temperate maize is attributable to greater stress tolerance. *Crop Sci.*, **39**, 1597–1604.

Willey, R. W. (1979): Intercropping – its importance and research needs. Parts I and II. *Field Crops Abstracts*, **32**, 1–10 and 73–85.

Wricke, G. (1962): Über eine Methode zur Erfassung der ökologischen Streubreite in Feldversuchen. *Z. Pflanzenzücht.*, **47**, 92–96.

Yates, F., Cochran, W. G. (1938): The analysis of a group of experiments. *J. Agric. Sci.*, **28**, 556–580.

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ANALYSIS OF THE MOISTURE CONTENT OF MAIZE KERNELS IN OVER-RIPE PLANTS

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The effect of varying weather conditions on the moisture content of the maize grain yield was investigated in Martonvásár, Hungary from late August to late September, and from the 3rd third of September to the 1st third of November between 1999 and 2002. In every year a close positive correlation (P=0.1%) could be observed between the moisture content in late September and the rate of drying down in October. Linear regression was used each year to determine the equilibrium moisture content, to which the moisture content of kernels returned if they contained less than this quantity of water in late September and harvesting was delayed. In the experimental years this value ranged from 15.24–19.01%.

Key words: maize, maturity group, drying down, remoistening, equilibrium constant

Introduction

In Hungary the economic viability of grain maize production is decisively influenced by the costs of artificial drying. Obtaining more detailed information on changes in the moisture content of the yield and on the relationships between modifying factors is thus an important task in breeding and crop production research.

During the ripening period, the drying down of maize kernels can be divided into two main periods (Hadi, 1982). Up till physiological maturity, the grain moisture content is determined by dry matter accumulation and the water management of the grain, which is partly regulated by physiological processes (Cavalieri and Smith, 1985). After the formation of the black layer water is lost from the kernels via the pericarp. Data presented by Purdy and Crane (1967) indicated that the pericarp thickness was decisive for the passive decline in the moisture content. Later research demonstrated a close positive correlation between the rate of drying down and the number of husks (Cavalieri and Smith, 1985), the positioning of the husks (Troyer and Ambrose, 1971) and other morphological traits, such as husk size, and the moisture content of the cob and the ear stalk (Hadi and Szundy, 1988).

Correlations between drying down and environmental factors have long been investigated by maize scientists. Aldrich (1943) attributed changes in this parameter to temperature and rainfall and Hallauer and Russell (1961) to the air temperature. Among the meteorological factors, Gunn and Christensen (1965) attached importance to the effect of heat sum increments and Magari et al. (1997) to the rainfall quantity.

Experiments in which the performance of registered and experimental hybrids is compared with that of the current standards make up an important part of the maize research in Martonvásár. When investigating agronomic responses an important aspect is the drying down ability of the hybrids. The effect of late harvesting on the grain moisture content is analysed using long years of data.

Materials and methods

The responses of 20 hybrids were examined using results obtained between 1999 and 2002 (Table 1). The small-plot field trials were sown in a randomised block design between 27 April and 2 May. The net plot size was 1.4 × 6.0 m = 8.4 m². An analysis was made of the effect of various climatic conditions from late August to late September, and from the 3rd third of September to the 1st third of November on the moisture content of the yield. The grain moisture content of the hybrids was determined for five sample ears of each hybrid, in two replications. Two 100 g samples of shelled kernels per plot were dried to constant weight at 105°C. Regression analysis was used to determine correlations between the quantitative variables (Sváb, 1981). The equilibrium grain moisture content was calculated from the point where the regression straight line intersected the x axis (Marton, 2002).

Results and discussion

A positive correlation significant at the P=0.1% or P=1% probability level could be observed in all but one year (1999) between the grain moisture content in late August and the rate of drying down to the end of September (Fig. 1). However, the correlation coefficients indicating the strength of the relationship between the variables only revealed close correlations during this part of the vegetation period in years considerably drier and hotter than the many years' average (Table 2). In years with weather normal for the given location, or cooler and wetter than this, only loose, moderately strong correlations were found. This can be explained by differences in the development of hybrids with diverse genetic backgrounds, which is also influenced by meteorological factors, and by the genetically determined drying down capacity of the hybrids.

A positive, very close correlation (significant at the P=0.1% probability level) could be observed between the grain moisture content in late September and the rate of drying down to the end of October (Fig. 2). During the final section of the generative development period, changes in grain moisture content depended decisively on external environmental effects and on the grain moisture content at the beginning of this period.

Table 1
Maize hybrids included in the experiments (Martonvásár, 1999–2002)

Hybrid	FAO number
1. Mv 272 (HU)	280
2. Helga (US)*	290
3. Mv 277 (HU)	310
4. Damacorn (HU)	330
5. Pelican (CH)*	350
6. Norma (HU)	380
7. Stira (US)*	390
8. Furio (CH)*	390
9. Mv 355 (HU)	390
10. Mv NK 333 (HU)	390
11. Mv Majoros (HU)	430
12. Mv TC 434 (HU) (HU)	440
13. Gazda (HU)	450
14. Mv 444 (HU)	450
15. Maraton (HU)	450
16. Dunia (US)*	450
17. DK 493 (US)*	460
18. Nóra (HU)	500
19. Kámasil (HU)	510
20. Florencia (US)*	530

*Standard

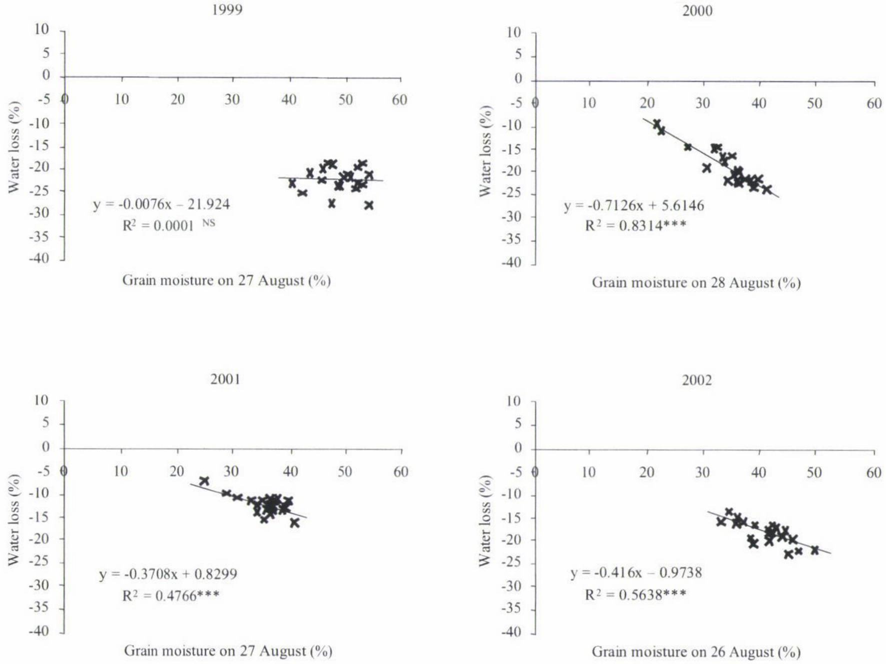

Fig. 1. Correlation between the maize grain moisture content in late August and the rate of water loss in September (Martonvásár, 1999–2002); NS: non-significant; ***: significant at the P=1% and P=0.1% probability level, respectively

Table 2
Meteorological data for the maize vegetation period (Martonvásár, 1999–2002)

Month	Rainfall (mm)					Mean temperature (°C)					Very hot days (t_{max}>30°C)			
	1999	2000	2001	2002	Mean*	1999	2000	2001	2002	Mean*	1999	2000	2001	2002
Apr	76	63	24	61	43	11.9	14.1	10.5	11.4	11.3	0	0	0	0
May	26	20	15	34	56	15.7	17.3	18.5	18.8	16.4	2	9	1	1
June	163	8	64	29	73	19.0	19.8	18.2	21.3	19.8	3	13	3	10
July	123	57	67	80	53	21.4	19.6	21.8	22.8	21.5	6	8	12	19
Aug	52	6	18	80	46	19.6	22.3	23.2	20.8	20.7	4	17	17	10
Sep	27	34	78	42	41	18.1	15.4	15.1	15.2	16.6	1	0	1	3
Oct	42	25	5	45	42	10.0	12.6	14.5	9.6	11.0	0	0	0	0
Apr–Oct	509	213	271	371	354	16.5	17.3	17.4	17.1	16.7	16	47	34	43

*30-year mean

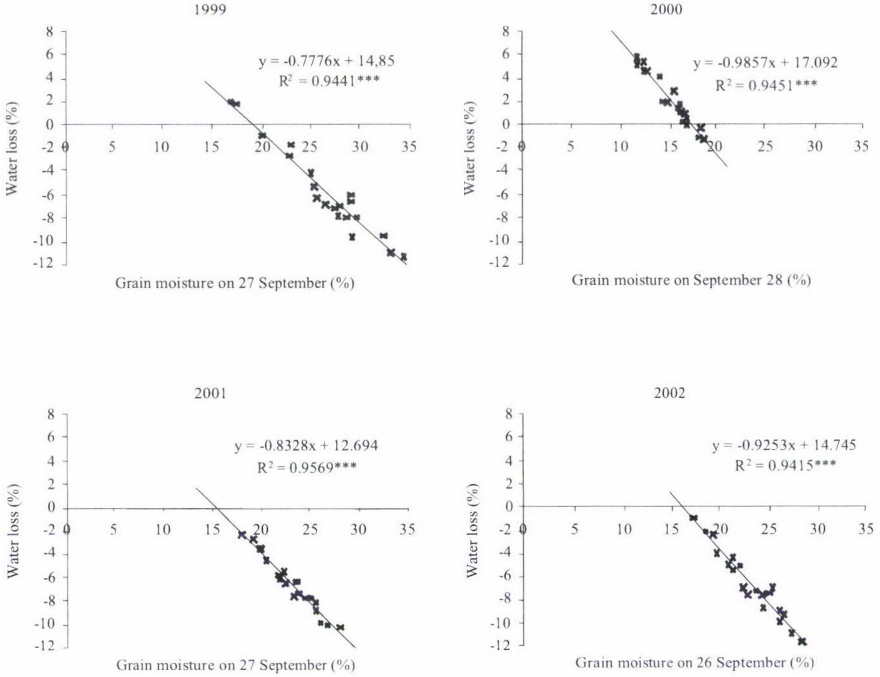

Fig. 2. Correlation between the maize grain moisture content in late September and the rate of water loss in October (Martonvásár, 1999–2002); ***: significant at the P=0.1% probability level

Based on the intersection between the regression straight line and the x axis, the equilibrium grain moisture content was calculated for each year. The values obtained for 1999–2002 were 19.01, 17.34, 15.24 and 15.94%, respectively (Fig. 2). This means that hybrids whose grain moisture content in late September was lower than the equilibrium value for the given year absorbed water during October. The higher the dry matter content of the yield, the greater the extent of remoistening. Only in 2001 and 2002 was no remoistening recorded for any of the 20 hybrids in Martonvásár.

The differences between the various maturity groups gradually decreased towards the end of the vegetation period, due to the drying of moister kernels and the remoistening of drier ones (Fig. 3). The grain moisture of hybrids in the FAO 300 or later groups declined significantly from late September to the 1st third of November in four of the years. In 2000, which was drier than average, the moisture content of the hybrids in late September was considerably below the equilibrium value, with the exception of a few genotypes, so late harvesting led to a significant increase in the likelihood that very early, early or mid-season hybrids would absorb moisture. The mean increase in moisture content in the individual maturity groups was 5.00, 2.82 and 1.21% in 2000 in the FAO 200, 300 and 400 groups, respectively.

Averaged over the years 1999–2002, moisture loss due to later harvesting increased as the vegetation period lengthened. The reduction in grain moisture content between late September and early November was 4.86% for hybrids in the FAO 500 group, 3.77% in the FAO 400 group and 1.80% in the FAO 300 group. The earliest (FAO 200) hybrids, however, exhibited a moisture surplus of 1.14% during this period.

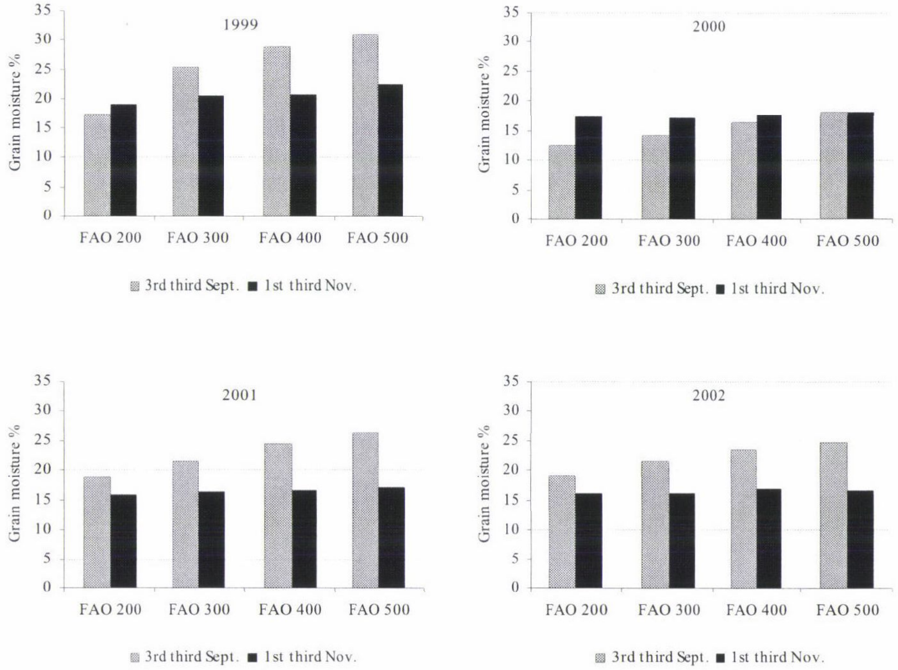

Fig. 3. Maize grain moisture content in the 3rd third of September and the 1st third of November for each maturity group (Martonvásár, 1999–2002)

The results achieved in different years confirmed that the genetically determined morphological traits of the hybrids had a decisive effect on the grain moisture content during the grain-filling period. After physiological maturity, however, although physiological and morphological differences between the hybrids were still important, changes in the grain moisture content were determined to a greater extent by meteorological factors.

The results indicate that when very early (FAO 200–299) hybrids with a short vegetation period are sown at the optimum date, their grain moisture in late September is lower than the equilibrium value in the majority of years. Due to the equilibrating processes reported by Cavalieri and Smith (1985), late harvest could cause an increase in moisture content under such conditions. At the same time the equilibrium values calculated for the various years suggest that the negative effects inducing remoistening can be expected to affect any kernels with a moisture content below 20%, irrespective of the genotype.

References

Aldrich, S. R. (1943): Maturity measurements in corn and an indication that grain development continues after premature cutting. *J. Am. Soc. Agron.*, **35**, 667–680.

Cavalieri, A. J., Smith, O. S. (1985): Grain filling and field drying of a set of maize hybrids released from 1930 to 1982. *Crop Sci.*, **25**, 856–860.

Gunn, R. B., Christensen, I. R. (1965): Maturity relationships among early to late hybrids of corn (*Zea mays* L.). *Crop Sci.*, **5**, 299–304.

Hadi, G. (1982): *A kukoricaszemek telítődése és vízleadása.* (Grain filling and drying down in maize.) M.Sc. Thesis. Martonvásár.

Hadi, G., Szundy, T. (1988): Ocenka rashtitelnykh shvojshtvu pri selektsii gibridov, ubrannikh pri nizkoi vlazhnosti. *Inform. Bull. Po Kuk.*, **7**, 27–40.

Hallauer, A. R., Russell, W. A. (1961): Effects of selected weather factors on grain moisture reduction from silking to physiologic maturity. *Agron. J.*, **53**, 225–229.

Magari, R., Kang, M. S., Zhang, Y. (1997): Genotype by environment interaction for ear moisture loss rate in corn. *Crop Sci.*, **37**, 774–779.

Marton, L. C. (2002): Yield, vegetation period and straw strength of maize hybrids. DSc thesis. Martonvásár.

Purdy, J. L., Crane, P. L. (1967): Influence of pericarp on differential drying rate in “mature” corn. *Crop Sci.*, **7**, 379–381.

Sváb, J. (1981): *Biometrical Methods in Research.* Mezőgazdasági Kiadó, Budapest.

Troyer, A. F., Ambrose, W. B. (1971): Plant characteristics affecting field drying rate of ear corn. *Crop Sci.*, **11**, 529–531.

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PARTICIPATORY MAIZE BREEDING IN PORTUGAL. A CASE STUDY

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Participatory maize breeding (PMB) was initiated in Portugal in 1984 by Dr. Silas Pêgo at Sousa Valley. The VASO project was intended to answer the problem facing small farmers, i.e. yield increasing without losing the parameters defined by farmers in polycropping systems maintaining the quality traits under a sustainable agriculture. This model is based on the Integrant Philosophy, which contrasts with the Productivist Philosophy. The Integrant Philosophy is intended to fit a multicrop agricultural system that corporate agriculture does not reach due to incipient market conditions. The present document intends to be a contribution to: 1) the study of 20 years of VASO; 2) methods used in PMB for Portuguese open-pollinated maize varieties and 3) present research.

Key words: participatory plant breeding, maize, open-pollinated varieties, VASO, Integrant Philosophy, on-farm breeding

Introduction

Twenty years have passed since the beginning of the Sousa Valley Project (VASO) in 1984. This paper is intended as a contribution to the evolution of maize breeding and genetic resources in Portugal, and intends to stress the importance of 20 years of participatory maize breeding (PMB) in the Portuguese Northern Sousa Valley region. Any description of VASO must be closely connected with Dr. Silas Pêgo, the founder of the Integrant Philosophy approach, which had its practical application through on-farm breeding. Pêgo also conducted the first basic implementation of the Portuguese Plant Gene Bank (BPGV). For a better understanding of these achievements some biographic data will be presented. An overview of this project will also be provided.

Silas Pêgo, the Man and His Work

Silas Pêgo is the kind of scientist who always thinks of science as a means to directly benefit farmers. His career, as well as his life, was early connected with maize. Born to Bento Fernandes Pêgo and Maria Esteves Pêgo in June 1942 in a small farming community in the extreme North of Portugal (Pias, Monção), he likes to say that he was born 50 m away from a maize field. A farmer's son, he grew up on a small farm in Minho province where polycrop systems are usual. These facts were crucial in his rethinking of the relationship between breeder and farmer. He graduated at Instituto Superior de Agronomia, Universidade Técnica de Lisboa in 1972. He started his professional career at Estação Agrária de Braga in its Núcleo de Melhoramento de Milho (NUMI) (maize breeding centre in Braga city). During his work at Braga he took several courses at DG/EAN (Genetics Department of National Agronomic Station, Oeiras) under the guidance of Professor Miguel Mota, who was responsible for the theory behind the Nutica population, a germplasm basis that Pêgo would use as a precursor of Fandango (one of the biggest ear-size germplasms in the world).

Later on, as director of NUMI, he laid the foundations of the future Portuguese Plant Gene Bank (BPGV), which was responsible for the Mediterranean Programme of FAO/IPGRI. He also organized and participated in several national and international germplasm collecting missions.

At NUMI he continued the research of António Lacerda, predecessor of Luís Freire de Andrade, his peer. He observed that some pure lines with fasciation expression showed several problems of stabilization. This problem was then used for his PhD research thesis in Plant Breeding and Cytogenetics at Iowa State University (ISU), USA, concluded in 1982 (Pêgo, 1982). The research developed by Pêgo under the supervision of Prof. Arnel Hallauer was a unique work done with Portuguese germplasm in the USA, and is still a hallmark for those who intend to work on maize fasciation (Pêgo and Hallauer, 1984). Before presenting his thesis he received a congratulatory mention from his advisor for his discovery of the U gene. As a scholar of The Rockfeller Foundation, before leaving the USA he obtained the permission of the foundation to extend the scholarship in order to discuss a maize breeding programme for Portuguese conditions with his former professors.

How to solve the problems facing small Portuguese farmers, where land is scarce and population density is high, i.e. where the American agriculture model is not appropriate and where the multinationals do not have a market to operate in, was another issue that encouraged him to conduct further research. From 1982 to 1985, Silas Pêgo was responsible for the Maize National Programme and, together with his mentor, Dr. Luís Costa Rodrigues, organized and constructed the National Breeding Programme, with two main components: 1) On-station approach, 2) On-farm approach, i.e. a Monoculture System (hybrid programme), adapted to the Productivist Philosophy, and Polycrop Systems (breeding populations), adapted to the Integrant Philosophy (Pêgo and Antunes, 1997).

The PMB Programme, as an Integrant Philosophy approach, was initiated in one of the best locations, side-by-side with Lousada farmers. The multidisciplinary scientific team attracted CIMMYT support from 1985 until Portugal joined the European Community.

Integrant Philosophy and Productivist Philosophy are not necessarily antagonists. Integrant Philosophy could be a very effective method of achieving diversity and germplasm for the Productivist Philosophy. According to the research done by Hallauer during the 70s and 80s, his populations began to be more productive than otherwise comparable commercial hybrids. The inbred lines obtained from these populations led to a new generation of better performing hybrids, i.e. from new improved populations it has been possible to extract superior inbred lines responsible for a continued rise in maize yield. Several authors (Altieri and Merrick, 1987; Brush, 1995; Bellon, 1996; Jarvis and Hodgkin, 1998; Sthapit et al., 2005) have focused on the importance of *in situ* conservation as a source of diversity to maintain a dynamic gene flow between germplasm conservation and breeding. This scientific rationality not only constitutes the basis for Pêgo's suggestion that the VASO project should be repeated in several regions of the country, but also stresses the importance of the pre-breeding approach, another of Pêgo's research topics, in which he developed some straightforward methods for germplasm evaluation. As Pêgo stresses, the importance of pre-breeding is related with the need to reduce the gap between "curators" and "breeders" or between "characterisation" and "utilisation". In fact, genebank catalogues represent a huge amount of data, as the IPGRI list of passport data parameters, but the most important ones for breeders, due to their direct relation with yield – inbreeding depression, combining ability and stress behaviour – are missing. If a breeder could afford to have even a preliminary evaluation of such parameters, this would allow him to screen a vast set of accessions for those with a better chance of success. Some examples of these proposed methodologies are discussed in Overlap Index Method (Moreira and Pêgo, 2003) and "HUNTERS" (Moreira et al., 2005a).

Integrant Philosophy and Participatory Maize Breeding

The *Integrant Philosophy* model, elaborated by Pêgo in 1983, was the approach used to tackle the reality facing, small farmers in Portugal where arable land is scarce and the population density is high. Under these small plot conditions the American model does not give an appropriate answer and the multinationals do not have attractive market conditions. The Integrant Philosophy approach takes into account not only the agricultural system, but also the farmer, as the most important genetic resource with the power of decision (Table 1). Pêgo's Integrant Philosophy is also the result of background interaction between: agriculture on small plots of land, the importance of genetic resources in breeding, an overview of maize in the world (FAO consultant), population improvement methodologies and the NUMI hybrid programme.

Table 1

Contrasting issues and/or consequences between the two philosophical models: productivist *versus* integrant (Pêgo and Antunes, 1997)

Contrasting factors	Philosophical model	
	Productivist	Integrant
1. Profession of faith	Yield is the determinant factor	Farmer's decisions are rational
2. Decisive centre	The seed (breeder)	The farmer
3. Dynamic action	Centripetal	Centrifugal
4. Energy	Fossil	Renewable
5. Raw materials	Exotic, inbreds	Local adapted populations
6. Science		
6.1. Gene action	Non-additive (heterosis)	Mainly additive
6.2. Breeding methods	Genealogical selection (+) biotechnology	Recurrent selection (-) biotechnology
6.3. Pathology	Resistance	Tolerance
6.4. Technology	(+) Mechanization (+) agrochemical (-) manpower and monocropping	(-) Mechanization (-) agrochemical (+) manpower and polycropping
7. Type of seed	Hybrid, uniformity	Open-pollinated, diversity
8. Final output	High yielding, quantity	Moderate yielding, quality
9. Environmental effects		
9.1. Protection level	Soil, water and air pollution	Soil, water and air cleanness
9.2. Genetic resources	Erosion	Conservation
9.3. Farming continuity	Leading to exhaustion	Sustainability

Materials and methods

VASO was implemented according to the Integrant Philosophy point of view. To achieve this goal three main decisions were taken: 1) The choice of location to represent the region, 2) the germplasm to start from, and 3) the farmer to work side-by-side with (Pêgo and Antunes, 1997).

Location

The Sousa Valley was chosen, taking into account the following factors: (a) Location in a traditional maize area characterized by polycropping systems, where maize still plays an important role, (b) One of the most fertile areas in the Northwest region of Portugal, (c) In 1985, 20–25% of its soils were planted with hybrids, compared with 15% as a national average. It was also on this area that the maize production (18 t/ha) champion was located (Mr. Coreolano), (d) The availability of a basic amount of agro/sociologic/economic data previously collected by members of the original multidisciplinary team provided the breeder with a systemic knowledge of the region, (e) The support of a local elite farmers' association (CGAVS) which agreed to be part of the project, (f) The possibility to test the efficiency of an alternative project expected to improve the local germplasm in order to be competitive, at least under certain specific circumstances, side-by-side with the local farmers.

Local germplasm

One of the pre-requisites of the Integrant Philosophy option (Table 1) was the existence of local adapted germplasm. This option respects the farmers' selection pursued over the last four centuries and also assures the environmental adaptation already achieved either for the soil/climate or for quality preferences. This assumption led to an extensive survey in the Sousa Valley Region, in the summer of 1984, looking for the best open-pollinated varieties (OPV) in the field. This survey allowed a reasonable choice of germplasm to start from: two OPVs were chosen, an early yellow flint variety (FAO 200) adapted to stress conditions (Al toxicity and water limitations) known as Amiudo, and a white flint medium maturity variety (FAO 300) with strong fasciation expression. Both varieties showed a high percentage of stalk and root lodging, like the great majority of landraces. Prior selection was made according to: second class soils (the first quality soils were already reserved for competitive hybrids), low nitrogen inputs, water limitations, flint type kernel, bread-making quality selected by the farmers, and polycrop system integration (maize-beans-*Lolium* sp.). This regional white flint OPV was named Pigarro, after an agreement between farmer and breeder.

Exotic germplasm

Fandango (FAO 600) is an open-pollinated selected composite derived from Nutica following the Design I crossing methodology. The Nutica broad population (FAO 700) was composed by intercrossing 76 yellow (dent and flint) elite inbred lines from the NUMI programme in natural isolation. In this set of 76 inbreds, 20% were Portuguese germplasm and 80% American germplasm.

The preparation of the material to be included in Nutica began in 1974. The Nutica Project was initiated in 1975 and finished in 1978.

In 1983, after Pêgo's return from the USA, the latest version of Nutica (now almost entirely yellow dent) was included in his program at ENMP (Elvas Breeding Station). In 1984, with the purpose of evaluating the gene action composition, the population was submitted to cross-pollination, type Design 1 (1 male crossed with 5 females), as part of the MSc project of Fátima Quedas under the supervision of Pêgo. The results obtained in the 2nd year trial were very promising, with high yielding levels obtained in the borders (composed by all the crosses in the trials). Due to the isolation conditions of the field, Pêgo used a mixture obtained in open pollination as a first basis of what would be designated as Fandango.

In 1985 Pêgo introduced Fandango in Lousada and stratified mass selection has been applied since then. This FAO 600 population, with yellow dent kernels, is characterized for having both high kernel row numbers (between 18 and 26) and large ear size. These characteristics explain why in each of the past 13 years, Fandango has been the winner of the contest "Best ear of Sousa Valley Region".

The farmer

Choosing the right people to work with is also a major decision in an on-farm project, where the work is carried out side-by-side with the farmer himself, to whom the power of decision will be delegated. All the information gathered was decisive for the choice of the two farmers. Their initial acceptance and enthusiasm to join the project turned out to be the best guarantee of success.

So, with careful respect for the local traditional agriculture, a deal was made with the farmers involved: while the breeder would apply his breeding methodologies, they should continue a parallel programme with their own mass selection criteria. With this tacit deal between breeder and farmer, three consequences became clear: 1) Respecting the "system" would imply accepting low input and intercropping characteristics, as well as accepting and respecting the local farmer as the decision maker, 2) With two simultaneous breeding programmes (the farmer's and the breeder's) the farmer would have a constant possibility to compare the effectiveness of both. This would allow the farmer to base his decisions on solid grounds, and 3) The option of diversity and quality as the first priority trait, due to starting from local adapted germplasm.

Breeding methodologies

In order to address both the yield component and pest and diseases problem, the breeding approach was to use quantitative genetics through population improvement selection, combining three main recurrent selection methodologies: phenotypic, S_1 and S_2 lines (Pêgo and Antunes, 1997).

Phenotypic recurrent selection

This methodology, involving mass selection with a two-parent control, is an improved extension of the common mass selection usually performed by all farmers (with only one parent control) and is the breeding tool lately used by the farmer, who has been advised to carry it out in a three-step sequence (A–B–C), the first two steps (A and B) in the field and the third one (C) during storage. The sequence follows this pattern:

Immediately before pollen shedding, selection is performed for the male parent by detasselling all the undesirable plants (pest and disease susceptible, weakest, plants that do not fit the desirable ideotype).

Some days before the harvest, besides selecting for the best ear size, the plants are kicked at their base (first visible internodes) to evaluate both their root and stalk quality. And, as an indirect measurement, the pest and disease tolerance can also be evaluated. In practical terms, if the plant does not resist the impact and lodges, it is eliminated. Moreover, special preference in selection is given to prolific plants.

In storage, after harvest, selection is performed separately for normal and prolific ears and always includes, besides ear length and kernel row number, prolificacy, and the elimination of damaged/diseased ears. The selected ears from both sets are finally shelled and mixed together to form the next generation seed.

Recurrent selection of S_1 and S_2 lines

Selection based on S_2 lines was initially the method to be applied to the two chosen regional germplasms (Pigarro and Amiudo) due to its good indication of the additive component of genetic variance ($3/2\sigma_a^2$) (Hallauer, 1992). Nevertheless, while Pigarro could be selfed well up to the S_2 stage, Amiudo exhibited such strong inbreeding depression that normal yield tests on S_2 lines became impossible. As a consequence, the yield tests were conducted on remnant S_1 seed according to the S_1 Lines Recurrent Methodology.

In the S_2 lines option, 1000 S_1 lines and then 500–600 S_2 lines were selected. The next step was the selection of 200 S_2 lines to be used in a yield trial, where 15 to 20% selection pressure was applied and a final set, i.e. 30–35 elite S_2s, was selected for the recombination season in order to form the first cycle seed (C1), and so on. During the selection process, the selection of plants to be selfed and selection before harvest led to the systematic elimination of diseased plants.

Results

Together with the on-farm project conducted in Seropédica in Rio de Janeiro State, Brazil (Machado and Fernandes, 2001), VASO started in 1984. Nevertheless, the VASO project in Lousada is probably the oldest PMB project in the world, because it has maintained, from the very beginning, different sets of germplasm identified and conserved under cold storage conditions. As an overall summary its output has resulted in the following improved populations: Pigarro (FAO 300 white flint), Amiudo (FAO 200 yellow flint), Aljezur (FAO 400 yellow flint), Aljezudo (FAO 300 yellow flint), Castro verde (FAO 600 yellow flint) and Fandango (FAO 600 yellow dent).

During the 2005 season, the evaluation of sets representing the distribution in time over the 20 years, cycles of phenotypic recurrent selection (Pigarro and Fandango) and S_2 lines recurrent selection (Pigarro) were carried out in three locations in Portugal and 5 locations in Iowa State, USA, and other evaluation sets are still underway. Nevertheless, some prior analyses have already been published (Pêgo and Antunes, 1997), yielding the following information:

1 - Pigarro produces tall plants with high ear placement and a high level of ear fasciation, responsible for a large number of kernel rows and consequently an improved kernel weight per plant.

2 - A gain of 17% (genetic and environmental) was registered when a comparison was made between $C_0$84 (7.0 t/ha) and $C_{1\text{-}}S_2$ (8.2 t/ha).

3 - Significant differences were detected between both C_1 and $C_0$86 and $C_0$90, but no significant differences were observed between the C_0s.

4 - The analysis of data on stalk and root lodging showed that the best yields depended on a combination of large ear size and good stalk and root characteristics.

5 - The evolution of phenotypic recurrent selection, from 1985 to 1990, did not lead to significant differences, but a positive tendency was registered (2% between $C_0$86–$C_0$84 and 2.4% between $C_0$90–$C_0$86).

6 - In plant quality and pest tolerance control, the farmer found somewhat contradictory results for root and stalk lodging between the first (84–86) and second (86–90) periods. This circumstance illustrates the communication and acceptance between farmer and breeder, discussed by Pêgo and Antunes (1997), and is a very interesting sociological testimony that stresses the importance of the breeder–farmer relationship and who really makes the decisions!

However, one major aspect of this project is linked with international evaluation. At the beginning of the PMB project in Sousa Valley, Dr. Wayne Haag (as CIMMYT director for maize breeding in the Mediterranean area), after having observed a Fandango population in the field, asked, "Where do we in America have an open-pollinated population like this, yielding 10 tonnes per ha?". As an immediate consequence, he decided to link CIMMYT with this project by supporting both its logistics and finances from 1985 until Portugal entered the European Economic Community (EEC).

In 2004 Professor Arnel Hallauer visited the project and after maize field observations he also mentioned in his report, "...In addition to reviewing the program with Dr. Silas Pêgo, I also had the opportunity to visit the farm of Mr. Francisco Ribeiro Meireles... Maize growth on the farm, and surrounding areas, looked very good. It seems good to excellent yields can be expected for that particular area".

Finally, the recent introduction of Pigarro in the central province of Huambo, through the initiative of the Angolan, governmental authorities, completes the picture. This improved open-pollinated white-flint variety – the preferred type of maize for food (Hallauer, 2004) – was chosen for its bread quality. Due to its good adaptation in the first year, multiplication facilities were built in Angola in order to supply small-scale African farmers to improve their living standard – one of the two aims for which VASO was born!

Discussion

From the beginning of VASO (1985) till the present time, the breeding process has been continued with the initial germplasm basis. The results presented in Pêgo and Antunes (1997), referring to breeding population methodologies that favour diversity and tolerance, indicate that non-adaptation to the competitive models of production imposed by the hybrid industry cannot be applied in all circumstances. It is strongly recommended that these two systems should work side by side because, besides giving a direct response to the problems facing small, quality-oriented, sustainable farming, the Integrant Philosophy also offers new germplasm sources for the hybrid industry, which is always eager for new inputs of improved genetic bases from which new inbreds can be extracted. In other words, the Integrant Philosophy could also be an important complement to the Productivist Philosophy, if more research on pre-breeding - an area that needs an effective approach between genetic resources and breeding – is done.

The VASO project suggests that this scientific approach should be replicated in several places in the country, especially in mountainous areas, where *in situ* conservation and sustainable quality-oriented agriculture could work together as part of a rural developmental policy, thus framing the economic basis for small-farming communities. As an extra output, new improved sources of quality-oriented germplasm could also serve the hybrid seed industry.

It is our opinion that, for the present and future, Portugal could play an important role in on-farm conservation, especially in white-flint maize, due to its traditional diet, probably unique in the world, based on maize bread ("Broa"). Even in the 21st century, maize could still have a say in the economic recovery of Portuguese organic farming. And if a greater role can be played in Africa, let the good news be spread to wherever it is needed!

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References

Altieri, M. A., Merrick, L. C. (1987): *In situ* conservation of crop genetic resources through maintenance of traditional farming systems. *Eco. Bot.*, **41**, 86–96.

Bellon M. R. (1996). The dynamics of crop intraspecific diversity: A conceptual framework at the farmer level. *Eco. Bot.,* **50**, 29–36.

Brush, S. B. (1995): *In situ* conservation of landraces in centres of crop diversity. *Crop Sci.*, **35**, 346–354.

Hallauer, A. R. (1992): Recurrent selection in maize. In: Janick, J. (ed.), *Plant Breeding Reviews*. John Wiley & Sons, Inc., New York, pp. 115–177.

Hallauer, A. R. (2004): Specialty corns. In: Smith, C. W. (ed.), *Corn: Origin, History, Technology, and Production*. John Wiley & Sons, Inc., New York, pp. 897–933.

Jarvis, D., Hodgkin, T. (1998): Strengthening the scientific basis of *in situ* conservation of agricultural biodiversity on-farm. Options for data collecting and analysis. *Proc. of a workshop to develop tools and procedures for in situ conservation on-farm*. 25–29 August 1997, Rome, Italy.

Machado, A. T., Fernandes, M. S. (2001): Participatory maize breeding for low nitrogen tolerance. *Euphytica,* **122**, 567–573.

Moreira, P. M., Pêgo, S. (2003): Pre-breeding evaluation of maize germplasm. The case of a Portuguese open-pollinated variety. In: A. R. Hallauer (ed.), Proceedings of the International Symposium on Plant Breeding. Mexico City, Mexico, 17–22 August, 2003.

Moreira, P. M., Santos, J. P., Simões, P., Santos, J. P., Vaz Patto, M. C., Carvalho, V., Pêgo, S. (2005a): Pré-avaliação de populações de milhos regionais da região centro. A utilização do método «HUNTERS». *II Colóquio de Melhoramento de Plantas e Conservação de Recursos Genéticos*. Santarém 18 de Novembro. Escola Superior Agrária de Santarém.

Moreira, P. M.; Santos, J. P., Simões, P., Santos, J. P., Vaz Patto, M. C., Carvalho, V., Pêgo, S. (2005b): Pré-avaliação de Populações de Milhos Regionais da Região Centro. Parâmetros Biométricos e Fitossanitários. *VII Encontro Nacional de Protecção Integrada*. 6 a 7 de Dezembro. Coimbra.

Pêgo, S. E. (1982): *Genetic potential of Portuguese maize germplasm with abnormal ear shape*. Ph.D. Dissertation. Iowa State Univ., Ames, Iowa.

Pêgo, S., Antunes, M. P. (1997): Resistance or tolerance? Philosophy may be the answer. *Proceedings of the XIXth – Conference of the International Working Group on Ostrinia*. Guimarães, Portugal.

Pêgo, S. E., Hallauer, A. R. (1984): Portuguese maize germplasm with abnormal ear shape. *Maydica*, **29**, 39–53.

Sthapit, B., Sajise, P., Jarvis, D. (2005): Community based on-farm conservation of agricultural biodiversity: Good practices and lessons learned from Nepal and Vietnam. *Second Colloquium on Plant Breeding and Plant Genetic Resources Conservation* organised by Portuguese Association of Horticulture (APH) in Santarem City, Lisbon, Portugal, 18 November 2005.

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HORMONE AND PHENOL LEVELS DURING GERMINATION AND OSMOPRIMING OF TOMATO SEEDS, AND ASSOCIATED VARIATIONS IN PROTEIN PATTERNS AND ANATOMICAL SEED FEATURES

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Seeds of tomato (*Lycopersicon esculentum* Mill., variety Castle Rock) were osmoprimed in polyethylene glycol 6000 (PEG; 20%) or K_2HPO_4 (200 mM) solution for 8 hours, 3 days or 7 days, while another group of seeds were left in water for the same periods. The GA_3/ABA ratio was the most important hormone factor, which promoted germination in seeds soaked in H_2O and led to improved germination performance. This ratio showed slight variations between hydroprimed and osmoprimed seeds after 8 hours, but afterwards, from 3 to 7 days, it was gradually increased in the osmoprimed seeds and was substantially elevated in seeds germinating in H_2O. Changes in the concentrations of phenolic compounds suggested their possible role in germination silencing in the osmoprimed seeds, but at relatively low concentrations. Protein patterns showed no marked variations in hydroprimed and osmoprimed seeds after 8 hours, but different types were observed, particularly after 7 days. A comparison of the protein banding patterns of seeds after 1 day and 7 days in the osmoconditioning solutions (PEG or K_2HPO_4), H_2O, GA_3 or ABA showed certain treatment-specific protein bands, particularly in PEG and ABA solutions. Longitudinal sections of seeds (after 3 days) showed lysis of the micropylar endosperm and radicle protrusion in H_2O or GA_3, whereas in PEG or K_2HPO_4 solution the radicle expanded inside the seed and the micropylar endosperm was completely intact. In ABA solution, the whole endosperm was compact and the seed became extensively desiccated.

Key words: tomato, polyethylene glycol, hydropriming, osmopriming, hormones, phenolics, gibberellic acid, abscisic acid, protein patterns, seed anatomy

Introduction

Seed priming is used as a technique for enhancing seed performance, notably with respect to rate and uniformity of germination (Taylor et al., 1998). Consequently, it improves seedling growth and enables better crop establishment (Job et al., 2000). The aim of seed priming is to achieve controlled water uptake by the seeds during the rapid water imbibitional phase up to the end of the following lag period (germination *sensu stricto*) phase (Bewley, 1997). Then, the primed seeds can be redried and sown without damage. It appears likely that priming enhances seed performance by initiating the early events of germination where the metabolic changes are below the threshold of cell division (Gurusinghe et al., 1999) and not enough to induce radicle protrusion (McDonald, 2000). The replication of DNA was also recorded to different extents during the priming of seeds of many plants (Bino et al., 1992; Lanteri et al., 1997; 2000), but appeared to be concomitant with cell cycle arrest in the G2 phase, with no mitotic activity (Gurusinghe et al., 1999). It has been established from studies on biochemical and molecular events during seed germination, that at earlier stages the embryonic axis initiates protein synthesis based on translation of the stored mRNAs (Srivastava, 2002). In this respect, the flux of genetic information is primarily based on translation and later on transcription-translation, which switches to the G1/S cell competence (Sanchez de et al., 1999).

Thus, the question is, how does the cell control which of the mRNAs in the seed will be engaged for translation. These control mechanisms are predicted to include cross talk regulation by growth hormones. Since these hormones are known to control the checkpoints to continuity of the germination program (Kigel and Galili, 1995; Bewley, 1997), they would also be predicted to interfere in the inhibition of visual germination in primed seeds. In this connection, growth regulators enhanced germination, as well as the emergence and growth of seedlings in many plants, either on addition to the priming solution (e.g. Cantliffe and El-Balla, 1994; Pill and Haynes, 1996; Grzesik and Nowak, 1998) or on being directly used as priming (pretreatment) solutions (e.g. Yoshiyama et al., 1996; Carter and Stevens, 1998; Upreti and Murti, 2000).

The interruption of seed germination during priming might also be controlled, besides ABA, by phenolic compounds, particularly phenolic acids (Li et al., 1993; Viémont and Crabbé, 2000). High levels of phenolics are also known to interfere in the control of seed dormancy (Weidner and Paprocka, 1997; Debeaujon et al., 2000), but at certain levels they exert a protective effect (Rice-Evans et al., 1997, Srivastava, 2002).

Several workers showed the enhancement of protein synthesis during the priming of seeds in many plants (McDonald, 2000). Higher levels of soluble proteins were observed at the expense of storage proteins (Job et al., 1997; Bourgne et al., 2000), β tubulin (Powell et al., 2000), some late embryogenesis abundant (LEA) proteins (Capron et al., 2000), heat shock proteins (Gallardo et al., 2001) and some enzymes (e.g. Wu and Fu, 1997; Bailly et al., 2000; Nascimento et al., 2001). Variation in gene expression programmes during germination and osmopriming has also been revealed recently by Barroco et al. (2005) and Soeda et al. (2005).

On the basis of the above, it was hypothesized that different signal transduction cascades might interfere to channel seeds left in water towards germination, and temporarily stop these processes in either hydroprimed (left for a limited period in H_2O, with germination stopped by subsequent drying) or osmoprimed seeds. To test this hypothesis, hormonal and phenolic changes, and the concomitant protein banding patterns, were followed after 8 hours, 3 days and 7 days in tomato seeds (variety Castle Rock) either soaked in H_2O, or osmoprimed in polyethylene glycol (PEG) or K_2HPO_4 for the same duration. In addition, changes in the protein patterns of the seeds in H_2O, osmopriming solutions, and GA_3 or ABA solutions were also assessed after one and seven days. Meanwhile, the anatomical features of these seeds were observed in microtome-prepared longitudinal sections after three days to observe changes in embryo features. In this connection, Hilhorst et al. (1998) and Welbaum et al. (1998) stated that tomato seeds represent a model system for the interaction between embryo expansive force and the weakening of enclosing tissues in controlling germination. They stated that the endosperm tissue enclosing the radicle tip, called "endosperm cap" or the "micropylar endosperm", requires weakening for radicle emergence and that such a process is primarily controlled by gibberellin (GA).

Materials and methods

A pure lot of seeds of tomato (*Lycopersicon esculentum* Mill. variety Castle Rock) was obtained from the Seed Technology Department, Horticulture Research Institute, ARC, Ministry of Agriculture, Giza, Egypt. The priming chemicals PEG 6000 and K_2HPO_4 and the growth hormones gibberellic acid (GA_3) and abscisic acid (ABA) were obtained from the Sigma-Aldrich Company.

Time course experiment

The osmopriming of Castle Rock seeds was carried out for 7 days, on the basis of a preliminary experiment (results not shown), in either polyethylene glycol (PEG) 6000 (20%) or K_2HPO_4 (200 mM) solution, as described in detail by Hegazi (2005). Samples were taken after 8 hours, 3 days and 7 days for the analysis of growth hormones and phenolic compounds, and the assessment of protein patterns.

For further investigation of the effects of GA_3 or ABA, protein patterns were compared in seeds after 1 day and 7 days in PEG, K_2HPO_4 (at the same concentrations mentioned above), H_2O, GA_3 (50 ppm), or ABA (10 ppm). Seed anatomy was also studied after 8 hours, 1 day, 3 days and 7 days. Sections taken after three days were found to present the most evident variations.

Extraction and estimation of growth hormones

The method of extraction was that adopted by Shindy and Smith (1975). IAA, GA_3 and ABA were estimated in the acidic ethyl acetate fraction after methylation according to Vogel (1975). A flame ionization detector was used for the identification and determination of acidic hormones, using a Hewlett Packard GC (5890) series. For the type of column, the programmed oven temperature and the gas flow rates, see Hegazi (2005). IAA, GA_3 and ABA standards were used. The cytokinin fractions (zeatin and zeatin riboside) were estimated in the aqueous phase after partitioning with saturated butanol. Detection was carried out using an HPLC isocratic UV analyzer with an ODS Hyparsil C_{18} column (Müller and Hilgenberg, 1986). For acidic hormones and cytokinins, the concentrations were automatically calculated.

Individual phenolic compounds

Seed samples were extracted twice, each for 24 hours, with methyl alcohol at 0°C as described by Diaz and Martin (1972). Determination was done after methylation, using HPLC. Various phenolics were compared to authentic samples.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) for protein analysis

Proteins were extracted from the seed samples in liquid nitrogen. The Tris buffer system used was that originally devised by Laemmli (1970), as described by Dunn (1993), with 2% (w/v) SDS and 5% (v/v) 2-mercaptoethanol to cleave the disulphide bonds. The slurry was centrifuged for 20 minutes at 12000 rpm. The samples were heated in a boiling water bath for 15 minutes before loading to ensure dissociation. Gel preparation was carried out according to Hames (1981), where 15% resolving gel was used. Bromophenol blue (0.001%) tracking dye was used for marking the buffer front during electrophoresis. The gel was electrophoresed at 25–30 mA constant current, then electrophoresed at 200 V (for about 8–9 hours). Staining was done using Coomassie brilliant blue, and a solution of 10% acetic acid and 45% methanol was used for destaining. In the quantitative analysis of the protein bands, laser densitometry was used to image the stained profiles.

Preparation of seed sections

Seeds were fixed in formalin acetic alcohol (FAA; 5: 5: 90). The paraffin method was followed for preparing longitudinal sections of seeds, as described by Johansen (1940). Microtome sections (8–16 microns thick) were prepared. Staining was done using gentian violet (in 50% alcohol) and erythrosine (in absolute alcohol). The slides were mounted with Canada balsam and microphotographs were taken using a light microscope.

Results and discussion

On the basis of preliminary experiments, the best benefits were observed when tomato seeds (variety Castle Rock) were primed for seven days in polyethylene glycol (PEG) 6000 (20%) or potassium hydrogen phosphate (K_2HPO_4; 200 mM) solution (at 25°C in darkness). In this respect, PEG performed better than K_2HPO_4 regarding germination potential, as well as for growth vigour and transplant uniformity (45-day-old stands). It was found that hydropriming was efficient for 8 hours, but seeds left longer showed visible signs of germination (radicle protrusion) after 3 days, whereas after 7 days the radicle had a measurable length (approximately 3 cm).

Changes in hormone levels

On the basis of the results (Fig. 1) it could be concluded that for seeds kept for from 8 hours to 7 days in water, the steep increase in the GA_3/ABA ratio seemed to correlate with the pattern obtained for germination. In this connection, the GA_3/ABA ratio might represent the main signal. This assumption was based on the results obtained and on the postulation that seed germination is regulated by a balance between the relative amounts of endogenous GA and ABA in the seeds and the sensitivity of their tissues to these hormones. This hypothesis has arisen from studies of seed germination in GA and ABA synthesis mutants and/or response mutants in tomato and *Arabidopsis* (Hilhorst and Karssen, 1992;

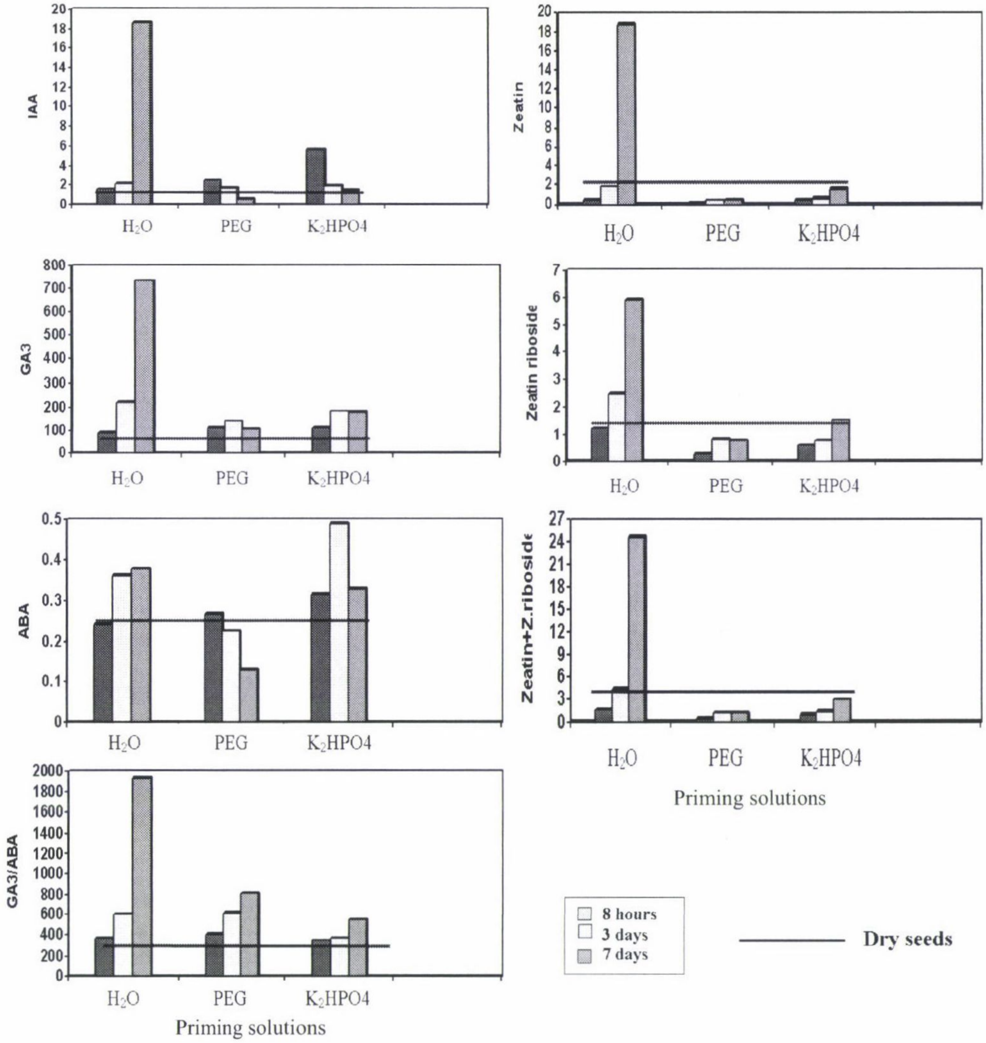

Fig. 1. Endogenous hormone concentrations (mg/100 g dry weight equivalents) of tomato seeds, variety Castle Rock, hydroprimed or osmoprimed with PEG (20%) or K_2HPO_4 (200 mM) for 8 hours, 3 days or 7 days. The control represents a dry seed lot. Each sample is the mean of 2 replicates

Ni and Bradford, 1993; Léon-Kloosterziel et al., 1996; Steber et al., 1998). On the other hand, the changes in IAA and cytokinins recorded in the present work might be the consequence of the biochemical and physiological shifts leading to embryo growth during hydropriming and the subsequent growth phase. On osmopriming with PEG or K_2HPO_4, the changes in hormone patterns after 8 hours and later (3 and 7 days) would indicate that certain signal transduction cascades take place in the quiescent seeds to stop germination proceeding. It is tentatively suggested that the drying of the seeds reverses the processes leading to germination, since the water potential is not sufficient, ABA is higher, and GA and/or cytokinins drop to below their genetically-determined threshold. The relatively low concentration of ABA measured in the present work, and the slight changes in this hormone as the priming time increases from 8 hours to 7 days (particularly with the more efficient priming solution PEG) suggest the general conclusion that ABA concentration is low or negligible when other hormones, particularly gibberellins, are relatively high, or in presence of a highly efficient osmoticum (Rock and Quatrano, 1995). In this connection, Kigel and Galili (1995) stated that ABA accumulation during embryogenesis maintains the embryo in developmental programme mode, but after these events are completed, the free ABA content in most seeds drops to a low or insignificant level, even if the seed is pushed towards quiescence. The results obtained also showed that a comparatively lower ABA content was observed after 7 days priming, particularly in PEG. This result might be supported to a certain extent by the conclusions of Xu et al. (1990) and Xu and Bewley (1995), that the prevention of ABA accumulation in the primed seed might mimic that occurring during germination. They added that either ABA at relatively low concentrations or an osmoticum can prevent germination, but only the osmoticum maintains protein synthesis.

Free phenolics

Other inhibitors of seed germination, besides ABA, include phenolic compounds (Li et al., 1993; Viémont and Crabbé, 2000). In the present work (Table 1), the simple phenols measured (benzidine, pyrogallol, catechol, caffeic acid and coumarin) increased either slightly or markedly in seeds primed in H_2O with the lapse of time from 8 hours to 7 days, with a substantial enhancement in their total values at the latest stage (7 days). In osmoprimed seeds (during the same period), hovewer, the changes recorded in individual phenolics were relatively slow, with a generally gradual increase in their total values from 8 hours to 7 days in treatments with PEG. In the case of K_2HPO_4, total simple phenols also gradually rose but with a maximum increment after 3 days. Thus, it was concluded that the relatively higher values of phenolic fractions in seeds in water after 3 and 7 days might be the consequence of germination and growth as a result of natural defence mechanisms and/or potential metabolism (Srivastava, 2002). In osmoprimed seeds, such relatively low and gradually increasing phenolics might concert with ABA in stopping germination at different rates, depending on the priming solution. It is suggested that such phenolics are required for seed protection during osmopriming, since other authors proved their benefits against solute leakage, imbibitional damage and oxidative stress during normal germination (Rice-Evans et al., 1997). It is also tentatively assumed that the relatively low levels of phenolics during osmopriming (as compared to the control) might be a seed strategy to sustain activity and avoid entrance into dormancy. According to Weidner and Paprocka (1997) and

Table 1

HPLC analysis of endogenous concentrations (mg/100 g d.wt.) of simple phenolic compounds in tomato seeds, variety Castle Rock, hydroprimed or osmoprimed for 8 hours, 3 days or 7 days. Priming solutions and their concentrations are listed. Seeds of the control are a normal (unprimed) dry lot

Priming solution	Time of priming	Concentrations					
		Benzidine	Pyrogallol	Catechol	Caffeic acid	Coumarin	Total value
H_2O	0 h	0.965	12.380	4.041	0.645	0.302	18.336
	8 h	1.056	4.712	3.638	0.762	0.492	10.66
	3 d	1.727	23.119	9.196	0.584	0.492	35.118
	7 d	1.898	52.972	42.24	1.785	1.849	100.744
PEG*	8 h	0.945	3.674	5.418	0.691	0.447	11.174
	3 d	0.967	5.548	6.365	0.614	0.416	13.91
	7 d	0.957	7.001	9.239	0.832	0.335	18.36
K_2HPO_4**	8 h	0.992	2.75	1.236	0.489	0.185	5.652
	3 d	1.447	8.48	14.04	0.293	0.197	24.457
	7 d	1.372	4.73	9.24	0.778	0.506	16.626

*20%; **200 mM

Debeaujon et al. (2000), high levels of free phenolics inhibit cell division and germination, and force the seed into dormancy. Variations in the concentrations of simple phenols in hydroprimed and osmoprimed seeds, measured by HPLC throughout the duration of the experiment, might be attributed to active transformations between different simple phenols, e.g. cinnamic acid, p-coumaric acid, coumarins, and the diphenol catechol and its product caffeic acid (Croteau et al., 2000).

Protein patterns during priming and post germination

The protein banding patterns of the Castle Rock variety were followed in seeds soaked in H_2O, as well as those osmoprimed in PEG or K_2HPO_4 for 8 hours, 3 days or 7 days (Table 2, Fig. 2). A comparison of the polypeptide profiles of the SDS-PAGE showed that after 8 hours, similar protein bands (having the same molecular mass) were generally observed, with very few exceptions, in seeds imbibing water or primed in one of the two osmotica used (PEG and K_2HPO_4). Slight changes were also achieved in the present work on extending the duration of priming to 3 days. During this period, osmopriming with PEG induced a positive treatment-specific protein band, the molecular mass of which was 55.09 kDa, while a protein having a molecular mass of 52 kDa was repressed in response to the same osmoticum (PEG). The induced protein (55.09 kDa) was also retained after 7 days priming in PEG. This protein was tentatively suggested to represent an HSP60-type protein (molecular ranges from 55–60 kDa; Bray et al., 2000). Generally, the transcripts of HSPs are lost if the water supply is continued (normal germination), but if it is limited, as in the case of osmopriming, their genes are reactivated in order to protect the quiescent seed against desiccation and maintain the structural integrity of the macromolecules (Srivastava, 2002). Since it was found that osmoprimed tomato seeds gave the

Table 2

Relative molecular weights and band concentrations (% protein) of the SDS-PAGE separated proteins of tomato seeds, variety Castle Rock, hydroprimed or osmoprimed with polyethylene glycol (PEG) or K_2PO_4, for 8 hours, 3 days or 7 days

Mr (kDa)	Marker	H_2O				PEG			$KHPO_4$		
		0 Hour	8 Hours	3 Days	7 Days	8 Hours	3 Days	7 Days	8 Hours	3 Days	7 Days
101.00	100.00										
86.79		5.00	8.24	6.65		31.89	13.04	9.55	7.02	18.60	
83.00	100.00										
77.87			17.93	13.46					20.50	48.11	
70.06			17.41	12.84		43.80	25.95				
59.08			12.36	12.36		31.81	17.89		11.44	14.13	
55.09							50.54	49.46			
52.00			10.00	7.90	9.73	20.15			9.09	11.20	31.92
50.60	100.00										
49.07		2.78	6.52	5.23	5.55	18.05	16.07	6.61	13.85	7.16	18.19
46.58		9.27	12.71	13.92		11.64	10.36	7.49	12.60	10.25	11.76
44.20		7.51	11.44	9.03	1.25	8.62	14.79	9.19	13.64	14.78	9.74
42.64			46.55	53.45							
40.64					7.66	17.50	12.38	10.31	7.84	13.39	30.92
37.87		3.04	11.09	10.33		13.42	16.64	11.25	8.97	12.91	12.35
36.50		6.48	11.93	11.34		12.06	13.29	11.84	10.71	12.38	9.98
35.50	100.00										
32.90		8.77	13.11	12.08	1.78	10.47	10.64	9.80	11.89	12.78	8.69
31.00		5.49	9.72	7.91		12.35	13.28	13.28	10.60	10.71	16.66
29.10	100.00										
28.09		32.91			67.09						
26.15		5.40	10.28	8.41		11.10	12.56	9.50	12.49	12.25	18.01
22.35		5.48	15.60	18.55	3.17	9.81	10.90	11.76	8.25	8.41	8.07
21.00		15.85				10.96	12.18		20.98	21.21	18.82
20.90	100.00										
14.70		7.10	9.97	12.30		10.12	11.94	12.59	10.00	12.39	13.59
12.88		8.87	10.40	10.27	13.92	5.96	6.64	6.18	12.53	12.89	12.33
7.40		7.00	10.65			10.65	12.97	17.54	10.65	12.97	17.54
Total bands	6	15	18	17	8	18	18	15	18	18	15

best performance when using PEG (Ismail et al., 2005), the induction of this 55-kDa protein might represent a molecular marker for the success of osmopriming Castle Rock seeds. Variations in metabolic trends after 7 days between seeds in H_2O and those in the priming solutions could be further proved by the patterns of seven protein bands (Mr: 46.58, 37.87, 36.50, 31.0, 26.0, 14.7, 7.4 kDa), which disappeared (after 7 days) in germinating seeds (in H_2O), but were still perceptible in osmoprimed seeds. It could be assumed, therefore, that the two small-molecular weight proteins mentioned above (Mr: 14.70 and 7.40 kDa) might represent smHSPs proteins (Gallardo et al., 2001), whereas the remaining five proteins might represent enzymes, antioxidants, receptors of silencing hormones such as ABA, etc. (Walker-Simmons and Goldmark, 1996). However, further work is required for the characterization of these proteins.

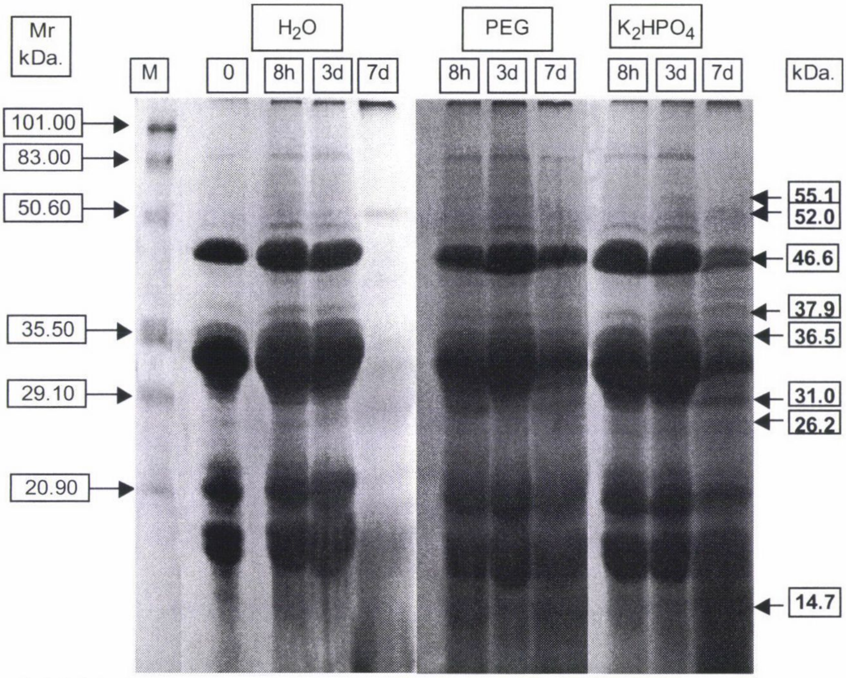

Fig. 2. SDS-PAGE profiles after Coomassie blue staining of proteins extracted from 'Castle Rock' tomato seeds hydroprimed (H_2O) or osmoprimed in 20% polyethylene glycol (PEG) or 200 mM K_2HPO_4 after 8 hours, 3 days or 7 days at 25°C in the dark. Protein bands of dry seeds (0) are also shown. The sizes of the molecular mass markers (M) are written on the left, while the arrows on the right mark the induction of a 55.1 kDa protein and the disappearance of a 52 kDa protein during osmopriming on PEG after 3 and 7 days. Six other marked protein bands ranging from 46.6-14.7 kDa are retained during osmopriming but disappear after 7 days in H_2O in young seedlings. The positions of the different proteins on the gel were checked using the Gel Documentation System software

Comparative effects of priming and treatments with GA_3 or ABA on protein patterns

A confirmatory experiment was designed in the last part of the present work on the basis of an immense body of literature which indicates the induction or up-regulation by GA_3 of many genes encoding the hydrolytic enzymes of stored food reserves, RNases, wall-digesting enzymes, the manipulation of cytosolic calcium $[Ca^{2+}]_{cyt}$ signals, etc., and/or the repression of α-amylase inhibitor (stored in dry seeds), alcohol dehydrogenase, and some HSPs or LEA proteins (Srivastava, 2002). Meanwhile, the majority of these genes are reversibly regulated by ABA. Thus, fine tuning for switching "on" or "off" for germination or silencing, respectively, would be adjusted via the interaction of the two hormones (Hilhorst and Karssen, 1992; Steber et al., 1998). Accordingly, this part has been devoted to comparing variations in the protein patterns in osmopriming solutions (PEG and K_2HPO_4), GA_3 (50 ppm) or ABA (10 ppm) with those in H_2O after 1 and 7 days.

The data obtained for protein banding patterns (Table 3, Fig. 3) can be summarized as follows:

1. After 1 day of imbibition, some protein bands showed common occurrence in hydroprimed and osmoprimed seeds (with both PEG and K_2HPO_4) as well as in GA_3- and ABA-treated seeds, and then completely disappeared after 7 days (Mr: 82.0, 77.9, 70.1, 51 kDa), when the seeds were at different physiological phases (young seedlings in H_2O or GA_3 solution and quiescent seeds or desiccated seeds in the priming solutions or ABA, respectively). Therefore, it seems reasonable to suppose that priming might enhance seed performance by inducing earlier events in germination, up to but not including radicle growth (Van Pijlen et al., 1995; McDonald, 2000), as well as activating DNA synthesis and the formation of the microtubular cytoskeleton (De Castro et al., 2000).

2. Certain protein bands disappeared after 7 days in GA_3 solution (Mr: 40.6, 12.9 kDa), in H_2O or GA_3 solution after 1 and 7 days (Mr: 37.9), and in H_2O or GA_3 solution after 7 days (Mr: 46.6, 42.6, 36.0, 26.2, 7.4 kDa). Thus, these proteins might represent markers of germination processes in tomato seeds of the Castle Rock variety. On the basis of the proteomic analysis of germinating and osmoprimed seeds, Gallardo et al. (2001) identified many proteins whose abundance was characteristic of 1-day hydroprimed seeds. This might also further indicate some similarity between seeds imbibing in H_2O or GA_3 solution.

3. Three unique ABA-specific protein bands (Mr: 86.8, 65.0, 8.4 kDa) were detected after 1 day of imbibition. This might verify the induction of certain proteins by this hormone, since their occurrence was not observed in the case of other treatments. In this connection, many ABA-inducible genes have been characterized, which were also upregulated, particularly in response to water stress (Shen and Ho, 1999).

4. At least five proteins (Mr: 37.9, 36.0, 28.1, 26.2, 7.4 kDa) were detected after 7 days in response to treatments with both osmotica (PEG and K_2HPO_4) as well as ABA. This might reveal some similarity between seeds imbibing in the osmotica and those in ABA solution.

The 28.1 kDa band might represent an osmotin-like protein that is known to be mainly induced by either water stress or ABA (Bray et al., 2000).

Anatomical features of the seeds in the presence of osmotica, GA_3, ABA and water (Fig. 4)

Embryo expansion in tomato is restricted by a brittle micropylar endosperm (Hilhorst et al., 1998; Welbaum et al., 1998; Bradford et al., 2000). A germination-specific endo-β-mannanase gene is expressed in this endosperm cap (Nonogaki et al., 2000) for the weakening of the endosperm cells. This enzyme hydrolyses the mannan backbone of galactomannan and (along with α-galactosidase and β-mannosidase) the products (galactose and mannose residues)

Table 3

Relative molecular weights and band concentrations (% protein) of the SDS-PAGE separated proteins of tomato seeds, variety Castle Rock, hydroprimed, osmoprimed with polyethylene glycol (PEG) or K_2PO_4, or treated with GA_3 or ABA, for 1 or 7 days

Mr (kDa)	Marker	1 day					7 days				
		H_2O 0.0	PEG 20%	K_2HPO_4 200mM	GA_3 50ppm	ABA 10ppm	H_2O 0.0	PEG 20%	K_2HPO_4 200mM	GA_3 50ppm	ABA 10ppm
101.00	100.00										
86.79						100.00					
83.00	100.00										
82.00		20.43	17.28	14.17	31.74	16.38					
77.87		20.73	17.88	13.91	30.41	17.07					
70.06		28.22	21.26		28.89	21.63					
64.99						100.00					
59.08						31.90		25.16		42.93	
55.09				59.33			40.67				
51.00		22.67	19.84	14.82	22.04	20.63					
50.60	100.00										
49.07		7.17	6.39	5.43	6.85	6.85	10.55	12.92	19.95	11.62	12.27
46.58		9.47	8.78	7.21	8.89	9.15		16.17	24.97		15.36
44.20		11.31	9.01	8.85	10.19	9.33	17.66	10.12	16.40	7.13	
42.64		15.41	9.85	11.17	12.78	13.89		9.90	16.22		10.79
40.64		17.83	15.82	15.41	16.08	12.92	5.10	3.52	3.34		9.96
39.35		9.54	8.87	10.18	13.60	10.45		15.30		13.04	19.02
37.87			11.27	13.73		12.51		24.21	14.97		23.31
36.00		21.93	11.30	7.31	16.64	14.62		11.97	8.33		7.89
35.50	100.00										
34.57		20.64	8.79	5.38	15.08	9.90		8.83	9.59	9.54	12.24
32.90				5.46	59.97		15.62	18.95			
31.49		20.22	17.42	18.66		16.56	3.94	2.44	11.25	2.29	7.24
30.00								32.40		33.12	34.47
29.10	100.00										
28.09								32.03	37.89		30.08
26.15		10.43	7.17	9.64	12.45	9.07		17.77	21.02		12.46
25.95				100.00							
24.27				35.46		64.54					
22.35		7.52	6.92	7.74	8.58	17.69	11.65	19.65	6.60	8.98	4.68
21.00		21.82	16.66	16.94	19.69				15.12		9.77
20.90	100.00										
19.19					59.42	40.58					
15.55		11.25	17.53	14.64	11.40	16.51			14.17	4.73	9.78
14.70							46.64			53.36	
12.88		11.86	9.76	9.21	14.20	9.85	5.75	14.54	13.82		11.01
10.47		17.97	15.30	15.58	21.11	16.10				13.94	
8.35						100.00					
7.40		18.41		14.89				24.79	23.23		18.68
6.67			48.56		51.44						
TB*	6	20	21	24	21	25	9	18	16	11	17

*Total bands

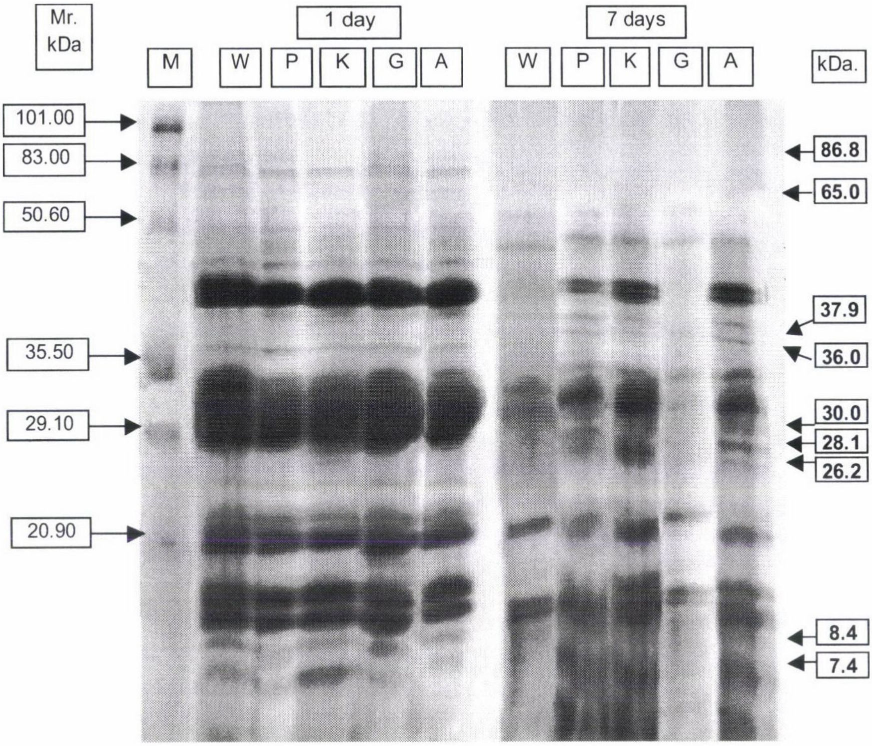

Fig. 3. SDS-PAGE profiles after Coomassie blue staining of proteins extracted from 'Castle Rock' tomato seeds primed in H_2O (W), 20% polyethylene glycol (P), 200 mM K_2HPO_4 (K), 50 ppm gibberellic acid (G) or 10 ppm abscisic acid (A) solution after 1 or 3 days at 25°C in the dark. The sizes of the molecular mass markers (M) are written on the left, while the arrows on the right mark the induction of three proteins (Mr: 86.8, 65.0, 8.4 kDa) after 1 day in (A) only. After 7 days, six protein bands (Mr: 37.9, 36.0, 30.0, 28.1, 26.2, 7.4 kDa) were induced in response to osmopriming with (P) or (K) as well as by treatment with ABA (A). The positions of the different proteins on the gel were checked using the Gel Documentation System software

are converted into sucrose and used for radicle growth (Srivastava, 2002). Bewley (1997) revealed that this enzyme is regulated by a hormone balance, where GAs (and/or cytokinins) move as a chemical signal from the embryonic axis via the cotyledons to the endosperm, where they induce *de novo* synthesis of the mannan hydrolytic enzymes. The same author also suggested that the process is repressed by ABA. Nascimento et al. (2004) also discussed a similar role of endo-β-mannanases in lettuce seeds.

Accordingly, longitudinal sections from seeds soaked for three days in H_2O, PEG, K_2HPO_4, GA_3 and ABA solutions were compared. In H_2O and GA_3 solution, the whole endosperm (micropylar endosperm, and lateral endosperm surrounding the embryo) was substantially loosened and lysed and the radicle showed marked protrusion through the micropyle. It was also obvious that the radicle exhibited cell division after being released from the micropyle, but within

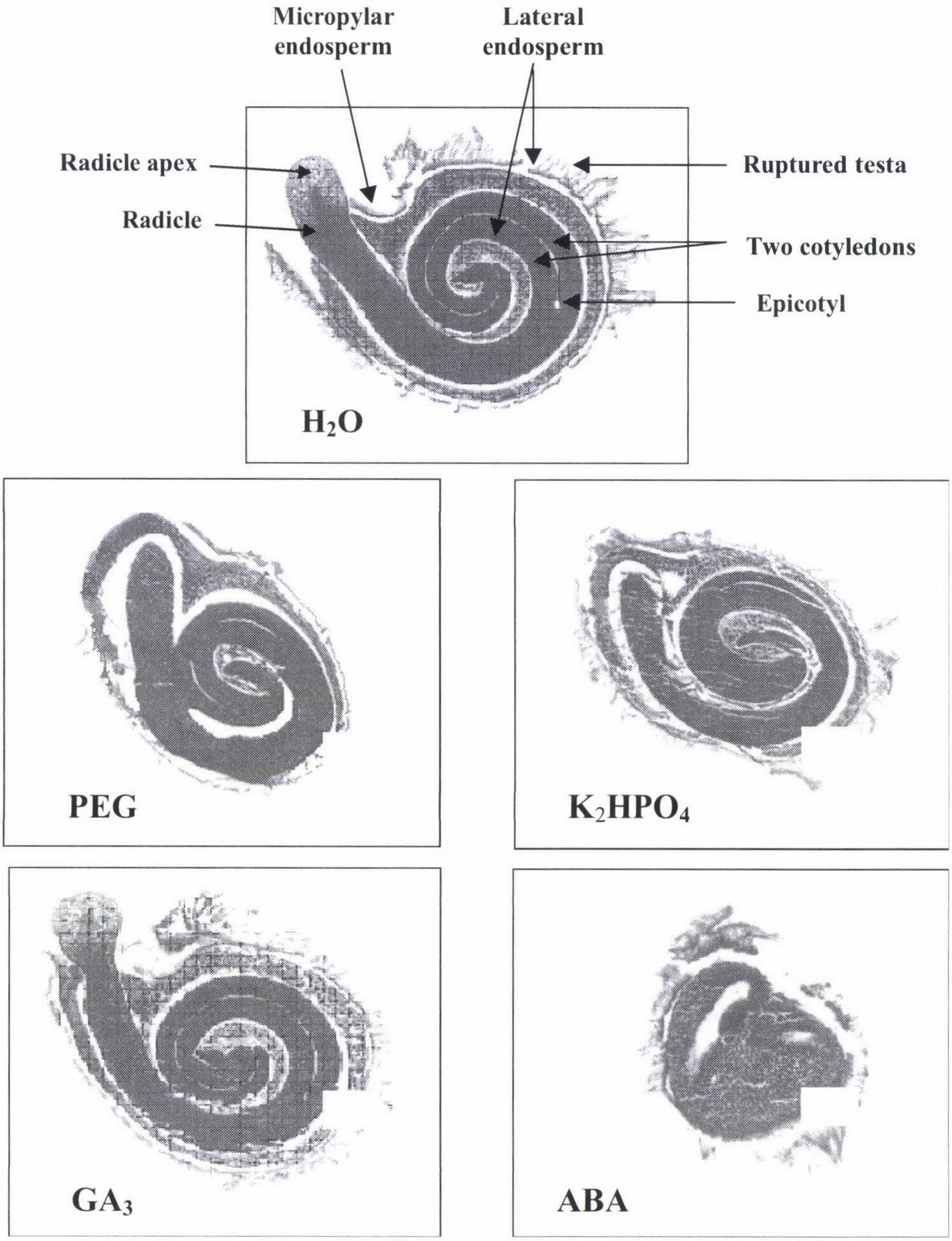

Fig. 4. Longitudinal sections of Castle Rock tomato seeds hydroprimed (H_2O), osmoprimed in 20% polyethylene glycol (PEG) or 200 mM K_2PO_4, or treated with 50 ppm gibberellic acid (GA_3) or 10 ppm abscisic acid (ABA) for 3 days. The figure shows the protrusion of the radicle from the lysed micropylar endosperm in water or GA_3. In the osmoprimed seeds (PEG and K_2PO_4) the micropylar endosperm represents a barrier against the expanding radicle. In ABA, the whole endosperm is compact

the seed tissue it apparently grew through expansion. This was supported by the results of Liang and Pardee (1992), who stated that the activities of the genes associated with cell enlargement proved that the initial enlargement of the embryo was due to water uptake and cell expansion, but typically did not involve cell division (Nonogaki et al., 2000). In the priming solutions (PEG and K_2HPO_4), however, the micropylar endosperm was still obstructing the expanding radicle (this was also evident after 7 days priming; results not shown). In ABA solution, on the other side, it was found during microtoming that the seeds were highly desiccated and it was difficult to discover any seeds with an intact endosperm. This desiccation was thought to mimic that which occurs in late embryogenesis.

Thus, it might be concluded that GA_3/ABA ratios can be manipulated (in Castle Rock tomato seeds) to permit the induction of the enzymes responsible for weakening the micropylar endosperm during germination (in H_2O and GA_3 solution), while the ratios experienced in osmoprimed seeds prevent germination from taking place. In this instance water uptake might have a regulatory function.

References

Bailly, C., Benamar, A., Corbineau, F., Côme, D. (2000): Antioxidant systems in sunflower (*Helianthus annuus* L.) seeds as affected by priming. *Seed Sci. Res.*, **10**, 35–42.

Barroco, R. M., van Poucke, K., Bergervoet, J. H. W., de Veylder, L., Groot, S. P. C., Inze, D., Engler, G. (2005): The role of the cell cycle machinery in resumption of postembryonic development. *Plant Physiol.*, **137**, 127–140.

Bewley, J. D. (1997): Seed germination and dormancy. *The Plant Cell,* **9**, 1055–1066.

Bino, R. J., DeVries, J. N., Kraak, H. L., Van Pijlen, J. G. (1992): Flow cytometric determination of nuclear replication stages in tomato seeds during priming and germination. *Ann. Bot.*, **69**, 231–236.

Bourgne, S., Job, C., Job, D. (2000): Sugarbeet seed priming: solubilization of the basic subunit of 11-S globulin in individual seeds. *Seed Sci. Res.*, **10**, 153–161.

Bradford, K. J., Chen, F., Cooley, M. B., Dahal, P., Downie, B., Fukunaga, K. K., Gee, O. H., Gurusinghe, S., Mella, R. A., Nonogaki, H., Wu, C-T., Yang, H., Yim, K-O. (2000): Gene expression prior to radicle emergence in imbibed tomato seeds. *Proc. 6th Int. Workshop on Seeds*, Merida, Mexico. pp. 231–251.

Bray, E. A., Bailey-Serres, J., Weretilnyk, E. (2000): Responses to abiotic stress. pp. 1158–1203. In: Buchanan, B., Gruissem, W., Jones, R. (eds.), *Biochemistry and Molecular Biology of Plants*. American Soc. Plant Biol., Rockville, MD, USA.

Cantliffe, J. R., El-Balla, D. N. (1994): Improving germination of carrot at stressful high temperature by seed priming. *Florida State Hort. Soc.*, **107**, 121–128.

Capron, I., Corbineau, F., Dacher, F., Job, C., Côme, D., Job, D. (2000): Sugarbeet seed priming: effects of priming conditions on germination, solubilization of 11-S globulin and accumulation of LEA proteins. *Seed Sci. Res.*, **10**, 243–254.

Carter, A. K., Stevens, R. (1998): Using ethephon and GA_3 to overcome thermoinhibition in 'Jalapeno M' pepper seed. *HortScience*, **33**, 1026–1027.

Croteau, R., Kutchan, T. M., Lewis, N. G. (2000): Natural products (secondary metabolites). pp. 1250–1318. In: Buchanan, B., Gruissem, W., Jones, R. (eds.), *Biochemistry and Molecular Biology of Plants*. American Soc. Plant Biol., Rockville, MD, USA.

Diaz, M., Martin, G. C. (1972): Peach seed dormancy in relation to endogenous inhibitors and applied growth substances. *J. Amer. Soc. Hort. Sci.,* **97**, 651–654.

Debeaujon, I., Karen, M., Kloosterziel, L., Koornneef, M. (2000): Influence of the testa on seed dormancy, germination, and longevity in *Arabidopsis*. *Plant Physiol.*, **122**, 403–114.

De Castro, R. D., van Lammeren, A. A. M., Groot, S. P. C., Bino, R. J., Hilhorst, H. W. M. (2000): Cell division and subsequent radicle protrusion in tomato seeds are inhibited by osmotic stress but DNA synthesis and formation of microtubular cytoskeleton are not. *Plant Physiol.,* **122**, 327–335.

Dunn, M. J. (1993): *Gel Electrophoresis: Proteins*. Bios Scientific Publishers, Oxford, UK. pp. 51–53.

Gallardo, K., Job, C., Groot, S. P. C., Puype, M., Demol, H., Vandekerckhove, J., Job, D. (2001): Proteomic analysis of *Arabidopsis* seed germination and priming. *Plant Physiol.*, **126**, 835–848.

Grzesik, M., Nowak, J. (1998): Effects of conditioning and hydropriming on *Helichrysum bacteatum* L. seed germination, seedling emergence and stress tolerance. *Seed Sci. and Tech.,* **26**, 363–376.

Gurusinghe, S. H., Cheng, Z., Bradford, K. J. (1999): Cell cycle activity during seed priming is not essential for germination advancement in tomato. *J. Exp. Bot.*, **50**, 101–106.

Hames, B. D. (1981): An introduction to polyacrylamide gel electrophoresis. pp. 1–91. In: Hames, B. D., Richwool, D. (eds.), *Gel Electrophoresis of Proteins. A Practical Approach.* IRL Press Ltd., Oxford, UK.

Hegazi, A. Z. A (2005): Physiological and Molecular Aspects of Seed Priming in Tomato. Pp. 49–50.

Hilhorst, H. W. M., Karssen, C. M. (1992): Seed dormancy and germination: The role of abscisic acid and gibberellins and the importance of hormone mutants. *Plant Growth Regul.,* **11**, 225–238.

Hilhorst, H. W. M., Groot, S. P. C., Bino, R. J. (1998): The tomato seed as a model system to study seed development and germination. *Acta Botanica Nederlandica*, **47**, 169–183.

Ismail, A. I., El-Araby, M. M., Moustafa, S. M., Hegazi, A. Z. A. (2005): Optimization of priming benefits in tomato (*Lycopersicon esculentum* Mill.) and changes in some osmolytes during the hydration phase. *Asian J. Plant Sci.*, **4**, 691–701.

Job, C., Kersulec, A., Ravasio, L., Chareyre, S., Pépin, R., Job, D. (1997): The solubilization of the basic subunit of sugarbeet seed 11-S globulin during priming and early germination. *Seed Sci. Res.*, **7**, 225–243.

Job, D., Capron, I., Job, C., Dacher, F., Corbineau, F., Côme, D. (2000): Identification of germination-specific markers and their use in seed priming technology. pp. 449–466. In: Black, M., Bradford, K. J., Vazquez-Ramos, J. (eds.), *Seed Biology: Advances and Applications*. CABI, Oxon, UK.

Johansen, D. A. (1940): *Plant Microtechnique*. New York Book Company, New York

Kigel, J., Galili, J. (eds.) (1995): *Seed Development and Germination*. Dekker, New York. pp. 231–234.

Laemmli, U. K. (1970): Protein gel preparation and staining. *Nature*, **227**, 680–685.

Lanteri, S., Belletetti, P., Marzach, C., Nada, E., Quagliotti, L., Bino, R. J., Hong, T. D. (1997): Priming-induced nuclear replication activity in pepper (*Capsicum annuum* L.) seeds. *Curr. Plant Sci. Biotech. in Agric.,* **30**, 451–459.

Lanteri, S., Portis, E., Bergervoet, W., Groot, S. P. C. (2000): Molecular markers for the priming of pepper seeds (*Capsicum annuum* L.). *J. Hort. Sci. Biol.*, **75**, 607–611.

Léon-Kloosterziel, K. M., van de Bont, J. A., Zeevaart, J. A. D., Koornneef, M. (1996): *Arabidopsis* mutants with reduced seed dormancy. *Plant Physiol.*, **110**, 233–240.

Li, H. H., Inoue, M., Nishimura, H., Mizutani, J., Tsuzuki, E. (1993): Interactions of trans-cinnamic acid, its related phenolic allelochemicals, and abscisic acid in seedling growth and seed germination of lettuce. *J. Chem. Ecol.,* **19**, 1775–1787.

Liang, P., Pardee, A. B. (1992): Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science*, **257**, 967–971.

McDonald, M. B. (2000): Seed priming. pp. 287–325. In: Black, M., Bewley, J. D. (eds.), *Seed Technology and its Biological Basis*. CRC Press LLC, Boca Raton, FL, USA.

Müller, P., Hilgenberg, W. (1986): Isomers of zeatin and zeatin-riboside in club root tissue, evidence for trans-zeatin biosynthesis by *Plasmodiophora brassica*. *Physiol. Plant*, **66**, 245–450.

Nascimento, W. M., Cantliffe, D. J., Huber, D. J. (2001): Endo-beta-mannanase activity and seed germination of thermosensitive and thermotolerant lettuce genotypes in response to seed priming. *Seed Sci. Res.*, **11**, 255–264.

Nascimento, W. M., Cantliffe, D. J., Huber, D. J. (2004): Ethylene evolution and endo-β-mannanase activity during lettuce seed germination at high temperature. *Scientia Agricola*, **61**, 156–163.

Ni, B. R., Bradford, K. J. (1993): Germination and dormancy of abscisic acid- and gibberellin-deficient mutant tomato (*Lycopersicon esculentum)* seeds. Sensitivity of germination to abscisic acid, gibberellin and water potential. *Plant Physiol.*, **101**, 607–617.

Nonogaki, H., Gee, O. H., Bradford, K. J. (2000): A germination-specific endo-β-mannanase gene is expressed in the micropylar endosperm cap of tomato seeds. *Plant Physiol.*, **123**, 1235–1245.

Pill, W. G., Haynes, J. G. (1996): Gibberellic acid during priming of *Echinacea purpurea* (L.) Moench seed improves performance after seed storage. *J. Hort. Sci.*, **71**, 287–295.

Powell, A. A., Yule, L. J., Jing, H. C., Groot, S. P. C., Bino, R. J., Pritchard, H. W. (2000): The influence of aerated hydration seed treatment on seed longevity as assessed by the viability equations. *J. Exp. Bot.*, **51**, 2031–2043.

Rice-Evans, C. A., Miller, N. J., Paganga, G. (1997): Antioxidant properties of phenolic compounds. *Trends Plant Sci.*, **2**, 152–159.

Rock, C. D., Quatrano, R. S. (1995): The role of hormones during seed development. pp. 671–697. In: Davies, P. J. (ed.), *Plant Hormones: Physiology, Biochemistry, and Molecular Biology*. Kluwer Academic Pub., Dordrecht, the Netherlands.

Sanchez, J. A., Calvo, E., Munoz, B. C., Orta, R. (1999): Comparison of seed conditioning treatments and their effects on germination in tomatoes, peppers, and cucumbers. *Cultivo Tropicales*, **20**, 51–56.

Shen, Q., Ho, T. H. D. (1999): Abscisic acid- and stress-induced promoter switches in the control of gene expression. pp. 187–218. In: Reynolds, P. H. S. (ed.), *Inducible Gene Expression in Plants*. CABI Publishing, New York.

Shindy, W. W., Smith, O. (1975): Identification of plant hormones from cotton ovules. *Plant Physiol.*, **55**, 550–554.

Soeda, Y., Konings, M. C. J. M., Vorst, O., van Houwelingen A. M. M. L., Stoopen, G. M., Maliepaard, C. A., Kodde, J., Bino, R. J., Groot S. P. C., van der Geest, A. H. M. (2005): Gene expression programs during *Brassica oleracea* seed maturation, osmopriming, and germination are indicators of progression of the germination process and the stress tolerance level. *Plant Physiol.*, **137**, 354–368.

Srivastava, L. M. (2002): Seed germination, mobilization of food reserves, and seed dormancy. pp. 447–471. In: *Plant Growth and Development: Hormones and Environment*. Academic Press, New York.

Steber, C. M., Cooney, S. E., McCourt, P. (1998): Isolation of the GA-response mutant *sly1* as a suppressor of *ABI1* in *Arabidopsis thaliana*. *Genetics*, **149**, 509–521.

Taylor, A. G., Allen, P. S., Bennett, M. A., Bradford, K. J., Burris, J. S., Misra, M. K. (1998): Seed enhancements. *Seed Sci. Res.*, **8**, 245–256.

Upreti, K. K., Murti, G. S. R. (2000): Osmotic stress-induced changes in seed germination and endogenous hormones in mepiquate chloride- and benzyl adenine-primed seeds of French bean. *J. Plant Biol.*, **27**, 259–264.

Van Pijlen, J. G., Kraak, H. L., Bino, R. J., De Vos, C. H. R. (1995): Effects of ageing and osmoconditioning on germination characteristics and chromosome aberrations of tomato (*Lycopersicum esculentum* Mill.) seeds. *Seed Sci., Tech.*, **25**, 303–310.

Viémont, J. D., Crabbé, J. (eds.) (2000): *Dormancy in Plants: From Whole Plant Behaviour to Cellular Control*. CABI Publishing, New York. 385 p.

Vogel, A. J. (1975): *A Text Book of Practical Organic Chemistry*, 3rd ed. English Language Book Society and Longman Group Ltd., London, UK. pp. 483–485.

Walker-Simmons, M. K., Goldmark, P. J. (1996): Characterization of genes expressed when dormant seeds of cereals and wild grasses are hydrated and remain growth-arrested. pp. 283–291. In: Lang, G. A. (ed.), *Plant Dormancy*. CABI Publishing, New York, USA.

Weidner, S., Paprocka, J. (1997): Preharvest sprouting as related to change in concentration of phenolic compounds in cereal grain and embryo sensitivity to phenolic acids during seed development. In: *Proceedings of COST 828 Workgroup*. Meeting, Barcelona, Nov. 10th, 1997. p. 2.

Welbaum, G. E., Shen, Z. X., Oluoch, M. O., Jett, L. W. (1998): The evolution and effects of priming vegetable seeds. A Symp. on Vegetable and flower seed quality, Boise, Idaho, USA, June 1998. *Seed Tech.*, **20**, 209–235.

Wu, X. Z., Fu, J. R. (1997): Priming effects of matriconditioning on *Brassica parachinesis* L. seeds. *Acta Scientiarum Naturalium Universitatis Sunyatseni.*, **36**, 69–73.

Xu, N., Bewley, J. D. (1995): The role of abscisic acid in germination, storage protein synthesis and desiccation tolerance in alfalfa (*Medicago sativa* L.) seeds, as shown by inhibition of its synthesis by fluridone during development. *J. Exp. Bot.*, **46**, 687–694.

Xu, N., Coulter, K. M., Bewley, J. D. (1990): Abscisic acid and osmoticum prevent germination of developing alfalfa embryos but only osmoticum maintains the synthesis of developmental proteins. *Planta*, **182**, 382–390.

Yoshiyama, M., Yajima, H., Atsumi, T., Esashi, Y. (1996): Mechanism of action of C_2H_4 in promoting the germination of cocklebur seeds. II. The role of C_2H_4 in the enhancement of priming effects. *Aust. J. Plant Physiol.*, **23**, 133–139.

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BIPLOT ANALYSIS OF GENOTYPE-ENVIRONMENT INTERACTION IN DURUM WHEAT USING THE AMMI MODEL

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The genotype by environment (GE) interaction is a major problem in the study of quantitative traits because it complicates the interpretation of genetic experiments and makes predictions difficult. In order to quantify GE interaction effects on the grain yield of durum wheat and to determine stable genotypes, field experiments were conducted with ten genotypes for four consecutive years in two different conditions (irrigated and rainfed) in a completely randomized block design with three replications in each environment. Combined analysis of variance exhibited significant differences for the GE interaction, indicating the possibility of stable entries. The results of additive main effect and multiplicative interaction (AMMI) analysis revealed that 12% of total variability was justified by the GE interaction, which was six times more than that of genotype. Ordination techniques displayed high differences for the interaction principal components (IPC1, IPC2 and IPC3), indicating that 92.5% of the GE sum of squares was justified by AMMI1, AMMI2 and AMMI3, i.e. 4.5 times more than that explained by the linear regression model. The results of the AMMI model and biplot analysis showed two stable genotypes with high grain yield, due to general adaptability to both rainfed and irrigated conditions, and one with specific adaptation.

Key words: durum wheat, adaptation, AMMI model, biplot analysis

Introduction

The genotype by environment (GE) interaction is a major problem in the study of quantitative traits because it complicates the interpretation of genetic experiments and makes predictions difficult. It is a particular problem in plant breeding where genotypes have to be selected in one environment and used in another (Kearsey and Pooni, 1998; Giauffret et al., 2000; Farshadfar and Sutka, 2003).

Various methods of GE interaction analysis exist, including parametric and non-parametric approaches. Parametric approaches are: (1) univariate analysis (regression analysis and stability variance analysis) and (2) multivariate analysis (principal component analysis, factor analysis, canonical component analysis, cluster analysis and biplot analysis) (Sharma, 1996; Roy, 2000; Chahal and Gosal, 2002).

The ordinary form of ANOVA is an additive model and therefore describes only the main effect (Snedecor and Cochran, 1989). Principal component analysis is a multiplicative model and has the opposite problem of not describing the additive main effects.

Linear regression models (Mandel, 1969; Finlay and Wilkinson, 1963) combine additive and multiplicative components and thus analyse both main effects and interaction, but in general they confound the interaction with the main effects (Wright, 1971), reducing its power for general significance testing. The additive main effects and multiplicative interaction (AMMI) model is a powerful multivariate method for multienvironmental trials (Romagosa and Fox, 1994).

This technique, also called FANOVA (Gollob, 1968), incorporates both additive and multiplicative components into an integrated, powerful least-squares analysis (Gauch, 1982; Voltas et al., 1999; Farshadfar and Sutka, 2003).

Plots showing both the genotypes and the environments simultaneously can be of great assistance in this respect, and are called biplots (Gabriel, 1971; Farshadfar and Sutka, 2003; Rubio et al., 2004).

The present investigation was carried out to quantify GE interaction effects on yield and to determine stable entries within the genotypic pool used in this study.

Materials and methods

Field experiments were conducted for four consecutive years (1998, 1999, 2000 and 2001) in two different conditions (irrigated and rainfed) at the College of Agriculture, Razi University of Kermanshah, Iran, giving a total of eight environments. The experimental layout for each environment was a completely randomized block design with three replicates. The environments were considered as random factors and the genotypes as fixed factors. The plots consisted of two 1 m rows spaced 20 cm apart. Ten genetically diverse durum wheat cultivars differing in their adaptation to rainfed environment conditions were used, together with Sardari, a landrace. The maximum and minimum temperatures were 44°C and –27°C and the average rainfall was 478 mm each year.

Combined analysis of variance, Bartlett's test of additivity, and mean comparison using Duncan's multiple range test were done using the statistical software MSTAT-C and SPSS (2206).

The additive main effect and multiplicative interaction (AMMI) analysis was performed using the model suggested by Crossa et al. (1990) as:

$$Y_{ij} = \mu + g_i + e_j + \sum_{n=1}^{h} \lambda_n \alpha_{ni} \gamma_{nj} + R_{ij}$$

where Y_{ij} is the yield of the i^{th} genotype in the j^{th} environment, μ is the grand mean, g_i is the mean of the i^{th} genotype minus the grand mean, e_j is the mean of the j^{th} environment minus the grand mean, λ_n is the square root of the eigen value of the principal component analysis (PCA) axis n, α_{ni} and $\gamma_n j$ are the principal component scores for PCA axis n of the i^{th} genotype and j^{th} environment, respectively, and $R_i j$ is the residual.

Combined analysis of variance was used to compute the first additive main effects of genotypes (G), environments (E) and the GE interaction.

A biplot based on the singular value decomposition (SVD) of GE contains only the GE interaction and can be referred to as a GE biplot. In contrast a biplot based on the SVD of G and GE contains only G plus GE, and will be characterized as a GGE biplot (Weikai et al., 2000). The GE biplots were projected for 23 lines tested at four environments. Clustering was computed for the genotype PCA score using an agglomerate hierarchical algorithm based on Ward's method (Farshadfar, 1998) and the result of cluster grouping for the genotype PCA score was projected in the biplot of PCA1 and PCA2, and the biplot of PCA1 and mean yield. The regression of yield for each variety on the yield means for each environment was computed and parameters MS-REG, the contribution of each variety to the regression component of the treatment × location (TL) interaction, and MS-TL, the contribution of each variety to interaction MS, were estimated with the IRRISTAT program.

Results and discussion

The results of combined analysis of variance (Table 1) showed significant differences for environments and the genotype × environment interaction, indicating the effect of the environment in the GE interaction, and as the GE interaction was significant, it was possible to proceed further and calculate phenotypic stability (Farshadfar and Sutka, 2003).

Tukey's test of additivity was not significant, leading to the conclusion that the effects are additive, hence the conditions required for analysis of variance are provided (Snedecor and Cochran, 1989).

Mean comparison (Table 2) revealed no significant differences between the genotypes, confirming the non-significance of the genotypes in the analysis of variance table.

The average grain yield of the lines ranged from 86.94 g in genotype 8 to 67.23 g in genotype 2.

The results of regression analysis (Table 2) revealed that the main effects of the GE interaction and genotypes were relatively small, accounting for 12% and 2% of the total sum of squares (TSS) in the GE matrix, respectively, while the effect of the environment was relatively high (0.86).

Table 1

Combined analysis of variance for grain yield under different rainfed and irrigated conditions

Source of variation	Degree of freedom	Mean square
Environment (E)	7	48981**
Error 1	16	219.13
Genotype (G)	9	1025.44ns
G × E	63	773.40**
Error 2	144	293.33
Non-additivity	1	540.7ns
Real error	143	295
Total	239	–

**: Significant at the 1% level of probability; ns: non-significant

Table 2

Regression analysis of durum wheat over different environments

Source of variation	Degree of freedom	Mean square	TSS%
Genotypes (G)	9	344.415ns	2
Environments (E)	7	16292.5**	86
G × E	63	255.705**	12
Source of variation	Degree of freedom	Mean square	G × E SS%
G × E (linear)	9	372.385ns	21
Deviation from regression	54	236.258**	79
Total	79	–	–

**: Significant at the 1% level of probability; ns: non-significant

Using regression analysis (Table 2), the GE interaction was divided into two components: linear and deviation from regression. The linear component was not significant, indicating the homogeneity of the regression coefficients and the disadvantage of regression analysis for stability discrimination. The GE interaction (linear) component accounted for 21% of the GE sum of squares (SSge). The mean square due to deviation from regression was highly significant and accounted for 76% of the SSge.

Stability analysis was performed using the regression model and the linear regression coefficient, the standard error, the interaction variance and the variance of deviation from regression were calculated for all the genotypes (Table 3).

Using the t-test it was concluded that genotype 9 was significantly different from unity, indicating specific adaptation for favourable conditions.

Table 3

Means, regression coefficient (bi), standard error (SE), GE mean square (MSge) and mean square deviation from regression (MSdev) of durum wheats under rainfed and irrigated conditions

Genotype	Mean	Bi	SE	MSge	MSdev
1	68.00	0.82	0.15	259.22	246.41*
2	67.03	0.83	0.09	131.12	100.94ns
3	79.35	0.90	0.14	206.46	223.95*
4	69.30	1.00	0.24	579.10	675.53**
5	70.24	0.82	0.08	118.84	79.13ns
6	78.65	1.16	0.14	231.66	220.99*
7	74.89	0.89	0.12	163.57	171.43ns
8	87.09	1.36	0.16	462.83	287.26**
9	78.78	1.16*	0.05	67.92	26.85ns
10	69.32	1.01	0.09	80.62	93.83ns

*, **: Significant at the 5% and 1% levels of probability, respectively; ns: non-significant

The regression coefficients were greater than unity for genotypes 6, 8 and 9, equal to unity for genotypes 4 and 10 and less than unity for genotypes 1, 2, 3, 5 and 7, indicating the presence of general adaptation for irrigated and rainfed conditions and specific adaptability for rainfed conditions, respectively (Farshadfar, 1997; 1998). When the mean squares due to deviation from regression were tested based on MSE_2/r, genotypes 1, 3, 4, 6 and 8 were significant, while genotypes 2, 5, 7, 9 and 10 were not significant, indicating greater phenotypic stability (Moghadam, 1994; Eberhart and Russell, 1966).

Genotype 9, with low S_{di}^2 and bi>1, had a desirable grain yield. Genotypes 2 and 5, with low S_{di}^2 and bi>1, had poor average yield. Genotype 10, with low, S_{di}^2, bi ~ 1 and average yield, was a stable genotype (Moghadam, 1994; Eberhart and Russell, 1966) with relatively poor general adaptability for irrigated and rainfed conditions (Lin et al., 1986; Lin and Binns, 1991).

AMMI model and pattern analysis

In the AMMI model, principal component analysis is based on the matrix of deviation from additivity or residual, while pattern analysis employs both classification and ordination techniques. In this respect not only the results of AMMI analysis but also the genotypes and environments can be grouped with respect to their similar responses (Alagarswamy and Chandra, 1998; Gauch, 1992).

Using ANOVA the yield sum of squares was partitioned into genotype, environment and GE interaction. Using principal component analysis the GE interaction was further partitioned. The results of AMMI analysis (Table 4) revealed that 12% of total variability was justified by the GE interaction, 86% by the environment and 2% by the genotype.

The contribution of the GE interaction is six times greater than that of the genotype, indicating the considerable effect of the GE interaction.

Table 4

AMMI analysis of adaptation in durum wheat

Source of variation	Degree of freedom	Mean square	TSS%
Genotype (G)	9	344.415	2
Environment (E)	7	16292.5**	86
G × E	63	255.705**	12
Source of variation	Degree of freedom	Mean square	G × E SS%
IPC 1	15	563.06**	52.5
IPC 2	13	284.22**	23
IPC 3	11	253.77**	17
Residual (Noise)	24	–	7.5
Total	79	–	–
Pooled error	160	285.91	–

**: Significant at the 1% level of probability

The ordination technique revealed high significant differences for IPC1, IPC2 and IPC3. The first interaction principal component (IPC1) explained 52.5% of the variability of GE, followed by IPC2 (23%) and IPC3 (17%).

Therefore, 92.5% of the GE sum of squares is justified by AMMI1, AMMI2 and AMMI3, which is 4.5 times more than that explained by the linear regression method. Even AMMI1 justified the GE interaction 2.5 times more than stability regression, displaying the relative efficiency of AMMI in relation to the regression model.

The values of interaction principal components (IPCA1, 2 and 3) for genotypes and environments are presented in Tables 5 and 6. The contribution of IPC1 to the GE interaction was greater than that of IPC2 and IPC3, the greatest interaction being found for genotype 4 and the least interaction for genotype 8.

The AMMI2 biplot (Fig. 1) explained 75.5% of the GE interaction, making it a useful test for interaction. It was observed that most of the genotypes and environments were dispersed around the biplot. Environments A, B, E and G had longer vectors and were further from the centre of the biplot. Environments C, D and F had shorter vectors, while environment H showed a length close to zero and hence had no GE interaction.

Table 5

Values of interaction principal components (IPC) for durum wheat genotypes

IPC3	IPC2	IPC1	Mean	Code
5.55895	0.696713	0.97302	68.00	1
1.549087	2.564502	1.130845	67.03	2
0.420854	4.296201	1.06141	79.35	3
0.045026	4.26427	5.650876	69.30	4
0.745608	1.856603	2.411613	70.24	5
0.02122	1.67596	3.66252	78.65	6
1.989206	1.235461	2.907633	74.89	7
2.687291	1.85994	5.28652	87.09	8
0.788411	2.36837	0.98374	78.78	9
2.64532	0.84095	0.13376	69.32	10

Table 6

Values of interaction principal components (IPC) for environments

IPC3	IPC2	IPC1	Mean	Code
0.73206	3.362232	0.70936	83.37	A
4.888042	3.813878	0.4403	60.18	B
2.22745	1.273044	1.608875	39.04	C
1.7115	0.515171	2.091738	13.7	D
0.54418	2.42299	8.28018	123.6	E
2.97652	1.11426	2.240324	68.46	F
3.369725	5.08103	3.256756	135.3	G
0.06605	0.34604	0.23215	70.40	H

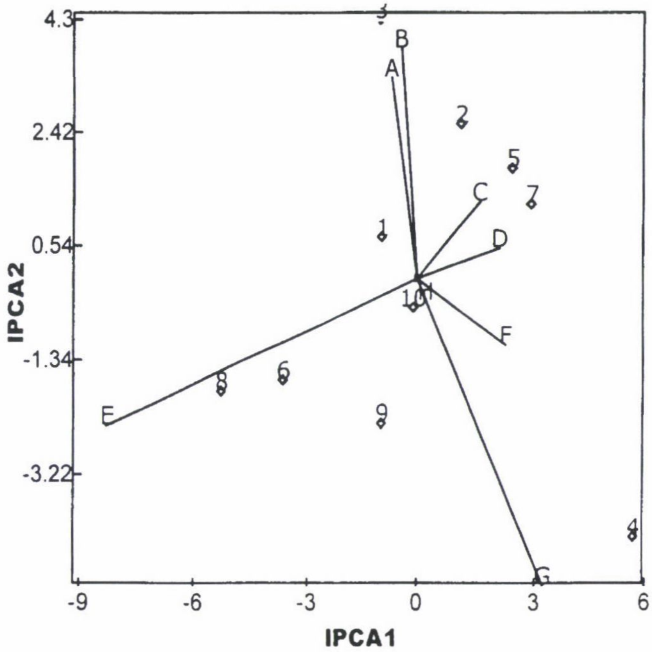

Fig. 1. Biplot analysis of the GE interaction for the AMMI2 model in durum wheat

Genotypes 1 and 10, with the least GE interaction, were located in the centre of the biplot, and hence had general adaptation for rainfed and irrigated conditions.

Genotype 8 had the longest vector for environment E, thus displaying specific adaptation for this environment.

Environments C, D and F were located on the side opposite environment E, hence they represent different environmental conditions. Genotype 8 had the most negative GE interaction with environment D. Genotype 4 had a negative interaction to environments A and B and specific adaptation to environment G, as environments A and B were on the side opposite environment G. Genotypes 2, 5 and 7 showed similar patterns, having the most negative interaction with environment E. Lines 6 and 9 also had similar patterns.

The general conclusion is that the test of non-additivity was not significant; hence, the block and treatment effects in the simple analysis of variance and the environment and treatment effects in the combined experiments were additive, i.e. the hypothesis of additivity of the model was true. The effect of the environment was large and covered most of the total sum of squares. The GE interaction was highly significant, so stable genotypes could be found by partitioning the GE interaction. The ratio of the GE interaction was six times that of the genotypes, indicating the importance of the GE interaction.

The results of regression analysis revealed that 21% of SSge was justified by the linear regression component. It should be mentioned that regression analysis is useful only when 50% of the GE interaction is justified by linear components and the variance of deviation from regression is equal for all the genotypes.

The efficiency of the AMMI model was 4.5 times greater than that of the regression model in explaining the GE interaction. AMMI2 was selected as the best AMMI model, so a biplot was constructed based on the AMMI2 model.

This biplot justified 75.5% of the variability of the GE interaction. Based on the AMMI2 model, genotypes 1 and 10 can be recommended as the most stable genotypes for rainfed and irrigated conditions.

References

Alagarswamy, G., Chandra, S. (1998): Pattern analysis of international sorghum multi-environment trials for grain-yield adaptation. *Theor. Appl. Genet.*, **96**, 397–405.

Chahal, G. S., Gosal, S. S. (2002): *Principles and Procedures of Plant Breeding*. Alpha Science International Ltd., Pangbourne, India. 149 p.

Crossa, J., Gauch, H. G. Jr., Zobel, R. W. (1990): Additive main effect and multiplicative interaction analysis of two international maize cultivar trials. *Crop Sci.*, **30**, 493–500.

Eberhart, S. A., Russell, W. A. (1966): Stability parameters for comparing varieties. *Crop Sci.*, **6**, 36–40.

Farshadfar, E., Sutka, J. (2003): Locating QTLs controlling adaptation in wheat using AMMI model. *Cereal Res. Commun.*, **31**, 249–255.

Farshadfar, E. (1997): *Plant Breeding Methodology*. Razi University Press, Kermanshah, Iran.

Farshadfar, E. (1998): *Application of Biometrical Genetics in Plant Breeding, Vol. 2*. Razi University Press, Kermanshah, Iran.

Finlay, K. W., Wilkinson, G. N. (1963): The analysis of adaptation in a plant breeding program. *Aust. J. Agric. Res.*, **14**, 742–754.

Gabriel, K. R. (1971): The biplot graphic display of matrices with application to principal component analysis. *Biometrika*, **58**, 453–467.

Gauch, H. G. (1982): Noise reduction by eigen vector ordinations. *Ecology*, **63**, 143–164.

Gauch, H. G. (1992): *Statistical Analysis of Regional Yield Trials. AMMI Analysis of Factorial Designs*. Elsevier, New York.

Giauffret, C., Lothrop, J., Dorvillez, D., Gouesnard, B., Derieux, M. (2000): Genotype × environment interaction in maize hybrids from temperate or highland tropical origin. *Crop Sci.*, **40**, 1004–1012.

Gollob, H. F. (1968): A statistical model which combines features of factor analysis and analysis of variance techniques. *Psychometrica*, **33**, 73–115.

Kearsey, M., Pooni, H. S. (1998): *The Genetical Analysis of Quantitative T*raits. Chapman and Hall, Boca Raton, FL, USA.

Lin, C. S., Binns, M. R. (1991): Genetic properties of four stability parameters. *Theor. Appl. Genet.*, **82**, 205–509.

Lin, C. S., Binns, M. R., Lefkovitch, L. P. (1986): Stability analysis: Where do we stand? *Crop Sci.*, **26**, 894–900.

Mandel, J. (1969): The partitioning of interaction in analysis of variance. *Mathematical Science*, **73**, 309–328.

Moghadam, M. (1994): *Advanced Plant Breeding*. Tabriz University Press, Tabriz, Iran.

Romagosa, I., Fox, P. N. (1994): Genotype × environment interaction and adaptation. In: Hayward, M. D., Bosemark, N. D., Romagosa, I. (eds.), *Plant Breeding Principles and Prospects*. Chapman and Hall, Boca Raton, FL, USA.

Roy, D. (2000): *Plant Breeding, Analysis and Exploitation of Variation*. Alpha Science International Ltd. Pangbourne, India. pp. 163–195.

Rubio, J., Flores, F., Moreno, M. T., Cubero, J. I., Gil, J. (2004): Effects of the erect/bushy habit, single/double pod and late/early flowering genes on yield and seed size and their stability in chickpea. *Field Crops Research*, **90**, 255–262.

Sharma, J. R. (1996): *Principles and Practice of Plant Breeding*. Tata McGraw-Hill Publishing Company Limited, New Delhi, India.

Snedecor, G. W., Cochran, W. G. (1989): *Statistical Methods*. Iowa State University Press, Ames., USA.

SPSS (2006): SPSS 13.0 for Windows, Data Analysis with Comprehensive Statistics Software. SPSS Inc., Chicago, USA.

Voltas, F. A., Sombrero, A., Lafarga, A., Igartua, E., Romagosa, I. (1999): Integrating statistical and ecophysiological analyses of genotype by environment interaction for grain filling of barley I. *Field Crops Res.*, **62**, 63–74.

Weikai, Y., Hant, L. A., Qinglai, S., Szalvnics, Z. (2000): Cultivar evaluation and mega-environment investigation based on the GGE biplot. *Crop Sci.*, **40**, 597–605.

Wright, A. J. (1971): The analysis and prediction of some two factor interactions in grass breeding. *J. Agric. Sci.*, **76**, 301–306.

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EFFECT OF WEED MANAGEMENT ON WEEDS, AND ON THE NODULATION, NITROGENASE ACTIVITY, GROWTH AND YIELD OF PEA (*Pisum sativum*)

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Effects of one pre-emergence herbicide (terbutryn/terbuthylazine) and one post-emergence herbicide (bentazone) along with unweeded and hand-weeded controls on weeds and on the nodulation, nitrogenase activity, nitrogen content, growth and yield of pea (*Pisum sativum*) were studied. Terbutryn/terbuthylazine was applied pre-emergence @ 1.40, 2.80 and 5.60 kg/ha whereas bentazone was sprayed 6 weeks after sowing @ 1.44, 2.88 and 5.76 kg/h. Terbutryn/terbuthylazine controlled all the weeds very effectively, whereas bentazone did not control some weeds such as *Polygonum aviculare*, *Poa annua* and *Elymus repens*. The herbicides decreased the number of nodules, the dry weight of nodules, the nitrogenase activity, the shoot dry weight, the nitrogen content in the straw and seeds, and the seed yield of peas, the effects generally being higher at higher rates of application. The adverse effects of herbicides on these parameters might be due to their effects on plant growth, as both the herbicides are known to adversely affect photosynthesis. Nitrogenase activity did not correlate well with plant-N content or shoot dry weight. However, there was a strong relationship between plant biomass and plant-N content, which suggests that researchers can rely on these parameters for studying the effects of treatments on nitrogen fixation, rather than measuring nitrogenase activity.

Key words: herbicides, nitrogenase activity, nodulation, weed control, weeds

Introduction

Weeds compete with crop plants for nutrients, moisture and light and thus reduce crop yield drastically. The magnitude of loss in yield depends upon the weed species, quantum of weeds and duration of crop–weed competition. To get higher yields of crops it is essential to control weeds at appropriate stage(s) with suitable method(s). In modern agriculture herbicides are widely used for effective and easy weed control. However, apart from controlling weeds, herbicides may also adversely affect crop growth. Poor plant growth due to the adverse effects of herbicides on plants may decrease the biological nitrogen fixation in legumes (Singh and Wright, 1999). Alternatively, the adverse effect of herbicides on legume–rhizobia symbiosis may decrease legume growth due to a decrease in biological nitrogen fixation. Under field conditions not only herbicides but the presence of weeds may also affect crop growth and biological nitrogen fixation in legumes, which also needs to be studied.

The acetylene reduction assay (Hardy et al., 1973; Turner and Gibson, 1980; Masterson and Murphy, 1980; Vessey, 1994) is a cheap, easy, rapid and sensitive method of determining biological nitrogen fixation indirectly by measuring nitrogenase activity. For pot-grown plants grown in a porous medium such as perlite or vermiculite, it is possible to measure the nitrogenase activity of undisturbed plants using either a flow-through gas system or a closed system. However, for field studies the roots have to be excavated for incubating in closed containers. Although researchers have criticised the use of the closed acetylene reduction assay (Minchin et al., 1983, 1986; Witty and Minchin, 1988; Minchin et al., 1994), no better methods, which are also cheap, have been developed for measuring the nitrogenase activity of field-grown plants. These workers have suggested the use of simple parameters such as plant dry weight, yield and total nitrogen, rather than nitrogenase activity, to determine the effect of given treatments. Hence, in the present studies, apart from measuring nitrogenase activity, the total nitrogen content in plants, the plant dry weight and the yield were also measured.

In this paper the effects of two herbicides, terbutryn/terbuthylazine and bentazone, on weeds, and on the nodulation, nitrogenase activity, growth and yield of pea are presented. These herbicides were chosen to represent two different chemical groups and timings of application. The herbicide terbutryn/terbuthylazine belongs to the chemical family ‘triazines’ and is applied pre-emergence, whereas bentazone is a ‘benzothiadiazole’ and is applied post-emergence. The studies also included unweeded and hand-weeded controls to separate the effects of herbicides and weeds on nitrogenase activity.

Materials and methods

Site description

The experiment was conducted at the Henfaes Farm, University of Wales, Bangor, Gwynedd on a soil of the Denbigh series. It was a medium-textured (sandy clay loam) mineral soil. The crop did not suffer for want of moisture at any of its critical stages of development.

Treatments and experimental design

The herbicide terbutryn/terbuthylazine was applied @ 1.40, 2.80 and 5.60 kg a.i./ha pre-emergence, whereas bentazone was applied @ 1.44, 2.88 and 5.76 kg a.i./ha post-emergence, 6 weeks after sowing. Terbutryn/terbuthylazine contains 350 g terbutryn + 150 g terbuthylazine/L and is available as Opogard 500 SC, whereas bentazone contains 480 g bentazone/L and is available as Basagran. Before spraying bentazone a pea leaf wax test (Gane et al., 1984; PGRO, 1993) was performed using a 1% solution of Crystal Violet to decide whether plants had sufficient leaf wax to enable the application of this herbicide without damaging the crop. The herbicides were sprayed with a knapsack sprayer fitted with a flat-fan nozzle using 800 litres of water/ha. There were hand-weeded and unweeded controls. In the unweeded treatment weeds were allowed to grow, whereas in the hand-weeded treatment weeds were removed manually with a trowel. Hand-hoeings in this treatment were done at 35, 50 and 64 days after sowing (DAS) and afterwards weeds were hand-pulled if and when necessary. Efforts were made to remove as many weeds as possible and thus at early stages of the crop this treatment had a negligible quantity of weeds. However, at later stages when the crop grew bigger it was not possible to remove weeds without damaging the crop plants, so in later stages the weeds, which were few in number and very weak, were not removed. The herbicides were very/fairly effective in controlling weeds but these plots still had some weeds, which were allowed to grow. The experiment was conducted in a randomised complete block design having four replicates.

Plant husbandry

The plots were established using an Oyjord drill with a row to row spacing of 12 cm. There were 10 rows in a plot, which measured 10 m × 1.2 m. Rex, a normal-leaved variety, was sown with a target plant population of 70 plants/m^2. Before sowing the seeds were not inoculated with *Rhizobium* culture, as sufficient rhizobia for pea are present in British soils and no inoculation is recommended (Gane et al., 1984). The seeds were treated with a fungicide (thiram) for protection against fungal diseases. The recommended rates of phosphorus and potassium, in the form of Champion Fertiliser (0-24-24) at the rate of 50 kg/ha each (as P_2O_5 and K_2O), were broadcast before drilling. No nitrogenous fertiliser was applied.

Weed data

Visual observations on the effects of herbicides on weeds and pea plants were recorded periodically on a whole plot area basis. Data on weeds were recorded 5, 10 and 14 weeks after sowing. Quadrat areas measuring 42 cm × 24 cm were removed and the number of plants of each weed species present were counted. Sampling for this purpose was done randomly and one quadrat area was harvested from each plot. At the first sampling, the weeds were very small and thus all weeds were combined and their dry weight recorded. For all other samplings the major weed species, namely *Polygonum aviculare,* was weighed separately and all other weeds were combined together. Dry weight was recorded after drying the samples in an oven at 80°C for 48 h.

Nodulation and nitrogenase activity

Nitrogenase activity was measured 7, 8, 10 and 12 weeks after sowing. These periods approximately corresponded to growth key codes of 104, 202, 204 and 207 as given by Knott (1987). Plants were dug up with the help of a trowel and/or spade from about 20–25 cm deep to recover roots and nodules. The roots were shaken lightly to remove soil, but were not washed. The plants were taken to the laboratory and the shoots were cut at the root/shoot interface. The unwashed whole root samples of five plants were then placed in 300 cm^3 glass bottles. The bottles were sealed with caps having a hole and fitted with rubber seals. With the help of a syringe 10% of the air was removed from the bottles and the same amount of acetylene was injected into the bottles. The samples were incubated for 30 min at 26°C, after which 0.5 cm^3 gas samples were collected in 1 cm^3 syringes for gas chromatography. The peaks separated by the gas chromatograph were recorded on graph paper with a chart recorder. Nitrogenase activity, expressed as μmol C_2H_4/plant/h (known as total nitrogenase activity), was calculated by the method of Jonsson (1988). After collecting the gas samples in syringes the roots were removed, washed with water and then dried with tissue paper. The nodules were detached, counted and weighed after drying in an oven at 70°C for 48 h. Dry shoot weight was also recorded.

Yield and yield components

At maturity 10 plants were randomly selected from each plot for the determination of number of pods/plant, pod length, number of seeds/pod, 1000-seed weight, and straw and seed weight/plant. Each whole plot was harvested with a Hege plot combine; the seeds were cleaned, dried at 80°C for 48 h and weighed, and the seed yield (t/ha) was calculated.

Nitrogen determination

The nitrogen percentage in the seed and straw was determined using the Kjeldahl method (AOAC, 1955) at final harvest. The nitrogen content in the plants was calculated by multiplying the nitrogen percentage values by plant dry weight.

Results

Visual observations on weeds

Terbutryn/terbuthylazine at all rates of application gave excellent control of all grassy and broad-leaved weeds and the plots were almost completely weedfree. The majority of the weeds did not emerge and those that emerged were soon killed. Bentazone killed most of the weeds, but *Polygonum aviculare, Poa annua* and *Elymus repens* were not killed completely even at the highest rate of application.

Weed count

Data on the number of individual weed species and the total number of weeds are presented in Tables 1 and 2, respectively. The weed species in the unweeded control included *Polygonum aviculare* (knotgrass), *Stellaria media* (common chickweed), *Sinapis arvensis* (charlock), *Spergula arvensis* (common spurrey), *Chenopodium album* (fat-hen), *Poa annua* (annual meadow grass), *Elymus repens* (common couch), *Fumaria officinalis* (common fumitory) and *Polygonum persicaria* (redshank). Data recorded 5 weeks after sowing show only the effect of terbutryn/terbuthylazine, since bentazone was applied 6 weeks after sowing and the first hand-weeding was done 5 weeks after sowing. Terbutryn/terbuthylazine at all the rates gave excellent control of grassy and broad-leaved weeds at all sampling dates. Bentazone controlled most weeds very well at all rates of application, except *Polygonum aviculare, Poa annua* and *Elymus repens.*

It was interesting to find a higher number of plants of some weed species in bentazone treatments compared to the unweeded treatment, indicating the uneven distribution of weeds in the field. The unweeded control shows the pattern of weed growth during the growing season. *Spergula arvensis* was observed only 5 and 10 weeks after sowing and by 14 weeks after sowing it disappeared. *Fumaria officinalis* was noticed only 5 weeks after sowing. *Polygonum persicaria* was observed only 10 weeks after sowing and before or after this period its appearance was not noticed.

Table 1
Effect of herbicides (kg/ha) on species-wise weed count (number of plants/m^2)

Weed species	Terbutryn/terbuthylazine			Bentazone			Unweeded	Hand-weeded
	1.40	2.80	5.60	1.44	2.88	5.76		
5 weeks after sowing[1]								
Chenopodium album	2	0	0	5	2	5	5	7
Elymus repens	0	0	2	0	2	0	2	0
Fumaria officinalis	0	0	0	0	0	0	2	0
Poa annua	0	0	0	2	2	2	5	0
Polygonum aviculare	17	10	0	131	117	171	107	141
Sinapis arvensis	2	0	2	20	7	12	17	12
Spergula arvensis	12	0	0	77	109	283	144	169
Stellaria media	2	0	0	47	52	74	47	35
10 weeks after sowing								
Chenopodium album	0	0	0	2	0	0	5	0
Elymus repens	1	0	0	7	6	8	3	0
Poa annua	9	0	0	60	49	74	29	0
Polygonum aviculare	0	0	2	104	55	84	106	0
Polygonum persicaria	0	0	0	0	0	0	10	0
Sinapis arvensis	0	0	0	2	0	0	15	0
Spergula arvensis	0	0	0	0	0	0	69	0
Stellaria media	0	0	0	0	0	0	35	0
14 weeks after sowing								
Chenopodium album	0	0	0	2	0	0	2	2
Elymus repens	0	0	0	2	0	12	0	22
Poa annua	0	2	0	25	32	87	25	0
Polygonum aviculare	0	2	0	62	20	117	102	0
Sinapis arvensis	0	0	0	2	0	0	12	0
Stellaria media	0	0	0	2	0	0	35	7

[1] These data show only the effect of terbutryn/terbuthylazine, as bentazone was applied 6 weeks after sowing (WAS) and in the hand-weeded treatment weeds were removed 5 WAS.

Data on the total number of weeds/m^2 (Table 2) show the excellent season-long control of weeds achieved with a pre-emergence spray of terbutryn/terbuthylazine at all rates of application. The application of bentazone resulted in a lower number of weeds at 10 than at 5 weeks after sowing. At 14 weeks after sowing bentazone at 1.44 and 2.88 kg/ha showed further decreases in the total number of weeds, but at 5.76 kg/ha the number of weeds increased.

In the unweeded treatment, compared to 5 weeks after sowing, the number of weed species decreased by 17 and 47% at 10 and 14 weeks after sowing, respectively. This decrease might be due to the weeds completing their life cycle and dying or to suppression by the crop.

Weed dry weight

Terbutryn/terbuthylazine-treated plots had negligible weed dry weight (Table 2). *Polygonum aviculare* was not only the major weed in number but in dry weight as well. At 10 weeks after sowing *Polygonum aviculare* accounted for 84, 92, 75 and 45% of the total weed dry weight, respectively, in bentazone treatments of 1.44, 2.88 and 5.76 kg/ha and in the unweeded control treatment. At 14 weeks after sowing the corresponding values were 76, 98, 49 and 68%.

Table 2

Effect of herbicides on total number of weeds and weed dry weight during the growing season of pea

Treatment	Rate (kg/ha)	Total no. of weeds/m^2	Dry weight of weeds (g/m^2)		
			Polygonum aviculare	Other weeds	Total
5 weeks after sowing[1]					
Terbutryn/terbuthylazine	1.40	37			0.09
	2.80	10			0.04
	5.60	5			0.12
Bentazone	1.44	283			7.79
	2.88	295			6.87
	5.76	548			12.57
Unweeded		330			5.53
Hand-weeded		365			7.19
S.E. (D.F. = 21)		157.5			3.320
10 weeks after sowing					
Terbutryn/terbuthylazine	1.40	10	0.0	0.0	0.0
	2.80	0	0.0	0.0	0.0
	5.60	2	0.0	0.0	0.0
Bentazone	1.44	176	21.2	4.1	25.3
	2.88	109	19.0	1.7	20.7
	5.76	166	15.0	4.9	20.0
Unweeded		273	45.7	56.8	102.6
Hand-weeded		0	0	0.0	0.0
S.E. (D.F. = 21)		55.7	11.78	9.08	19.23
14 weeks after sowing					
Terbutryn/terbuthylazine	1.40	0	0.0	0.0	0.0
	2.80	5	0.0	0.1	0.1
	5.60	0	0.0	0.0	0.0
Bentazone	1.44	94	10.4	3.2	13.6
	2.88	52	5.9	0.1	6.0
	5.76	216	16.1	4.5	20.6
Unweeded		176	29.8	9.2	39.0
Hand-weeded		32	0.0	2.0	2.0
S.E. (D.F. = 21)		59.5	7.38	4.00	10.75

[1] The data show only the effect of terbutryn/terbuthylazine, as bentazone was applied 6 weeks after sowing and in hand-weeded treatment weeds were removed the day after recording these data.

Visual symptoms on pea

Terbutryn/terbuthylazine caused chlorosis and even the death of some plants, and the effect was severe at higher rates of application. Observations showed that plants which had chlorosis following treatment with terbutryn/terbuthylazine at 1.40 kg/ha were almost recovered by 7 weeks after sowing and those with higher rates of application were recovering. Bentazone caused yellowing of the leaves and the effect was more marked as the rate of application increased. However, no mortality was observed in bentazone-treated plants.

Nitrogenase activity

The treatments did not differ significantly ($P>0.05$) in nitrogenase activity (Fig. 1). At 7 weeks after sowing, though differences between the treatments were non-significant, lower nitrogenase activity was observed at higher rates of application of both the herbicides. The adverse effect of terbutryn/terbuthylazine at the highest rate was far greater than that of the corresponding rate of bentazone. At other sampling dates there was no clear trend towards a decrease in nitrogenase activity with an increase in herbicide application. Though the differences were non-significant, the hand-weeded treatment had higher activity than the unweeded treatment at 8 and 10 weeks after sowing, but this trend was reversed at 7 and 12 weeks after sowing. The overall nitrogenase activity was relatively high at 7 and 8 weeks after sowing and then decreased, the decrease being very large at 12 weeks after sowing, when the plants were at the seed-filling stage.

Nodulation

A lower number of nodules/plant was recorded at higher rates of terbutryn/terbuthylazine, especially at 7 weeks after sowing (Fig. 2). At 12 weeks after sowing the proportion of pink nodules to total nodules was between 70 and 85% in different treatments. The hand-weeded treatment had the highest nodule dry weight/plant at all sampling dates (Fig. 3). Both herbicides had lower nodule dry weight/plant and the effect was generally more severe at the high rates of application. Of the two herbicides, terbutryn/terbuthylazine was more damaging in respect of nodule dry weight. Despite large differences the treatments did not differ significantly ($P>0.05$) in number of nodules and their weight, probably due to the large variability. The number and dry weight of nodules were the highest at 8 weeks after sowing and the lowest at 12 weeks after sowing.

Shoot growth

Shoot dry weight increased with the age of the plants (Table 3). The treatments significantly ($P<0.05$) influenced shoot dry weight/plant at 7, 8 and 10 weeks after sowing (Table 3). Terbutryn/terbuthylazine at 5.60 kg/ha and bentazone at 5.66 kg/ha at 7 weeks after sowing, all herbicide treatments except terbutryn/terbuthylazine at 2.80 kg/ha at 8 weeks after sowing, and all herbicide treatments at 10 weeks after sowing resulted in significantly lower shoot dry weight/plant compared to the hand-weeded treatment.

Yield and yield components

None of the treatments had a significant ($P>0.05$) effect on number of pods/plant, pod length, number of seeds/pod, 1000-seed weight or seed yield (Table 4). However, seed yield tended to decrease with higher rates of both the herbicides. The unweeded treatment yielded 10.6% less than the hand-weeded treatment.

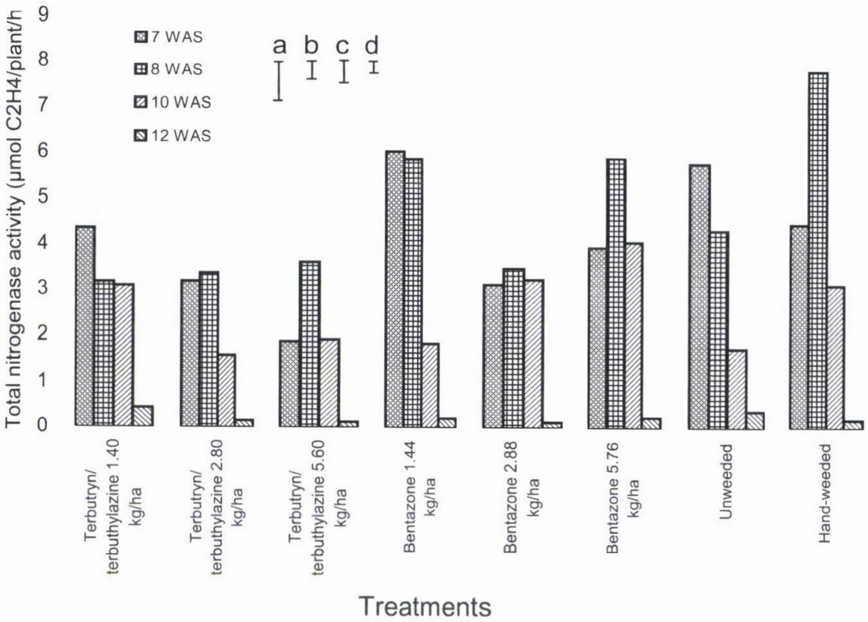

Fig. 1. Effect of different treatments on total nitrogenase activity at different weeks after sowing (WAS) in peas (a, b, c and d indicate SE of the means [21 df] at 7, 8, 10 and 12 WAS, respectively)

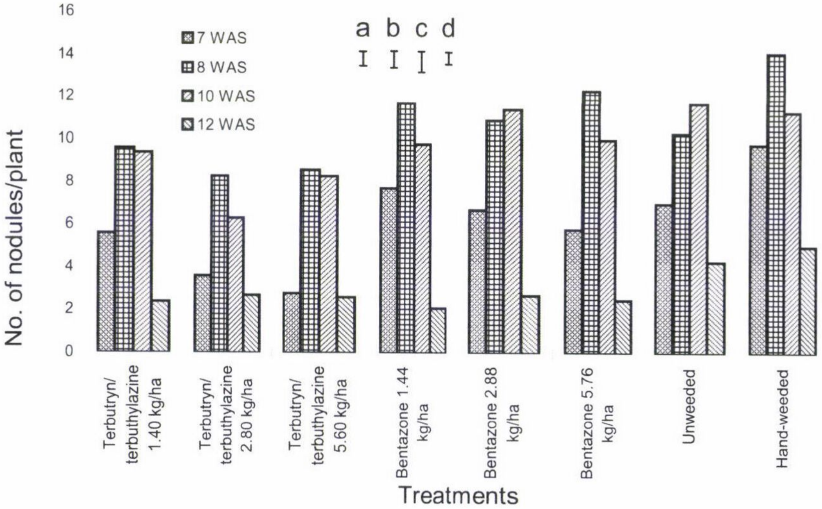

Fig. 2. Effect of different treatments on number of nodules per plant at different weeks after sowing (WAS) in peas (a, b, c and d indicate SE of the means [21 df] at 7, 8, 10 and 12 WAS, respectively)

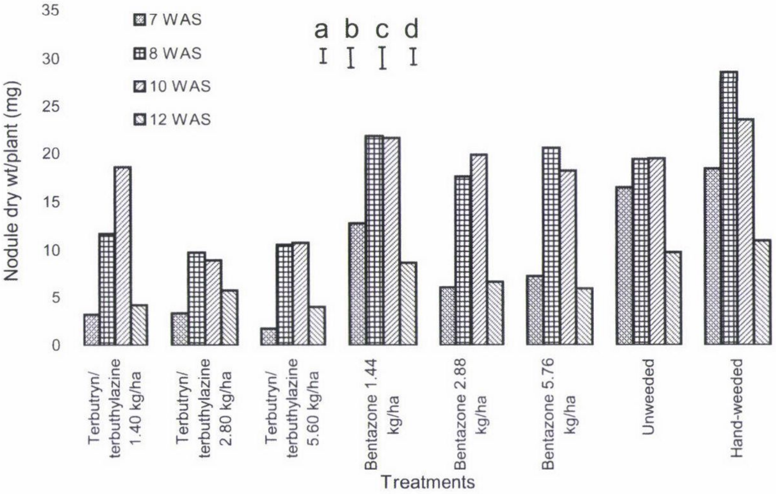

Fig. 3. Effect of different treatments on dry weight of nodules at different weeks after sowing (WAS) in peas (a, b, c and d indicate SE of the means [21 df] at 7, 8, 10 and 12 WAS, respectively)

Nitrogen content

Nitrogen content was not influenced significantly (*P*>0.05) in either the seed or the straw (Table 5). However, the nitrogen content in the straw and seed and the total nitrogen in the plant were lower in all the herbicide treatments and in the unweeded control than in the hand-weeded treatment. Compared to the hand-weeded treatment, the unweeded control had 34, 18 and 33% lower nitrogen content in the straw, seed and whole plants, respectively.

Table 3

Effect of herbicides on shoot dry weight (g/plant) of pea plants

Treatment	Rate (kg/ha)	Weeks after sowing					
		7	8	10	12		
					Shoot		
		Shoot	Shoot	Shoot	Stem+leaf	Pods	Total
Terbutryn/terbuthylazine	1.40	2.86	4.41	9.96	9.64	4.91	14.55
	2.80	2.30	4.95	8.21	10.38	4.74	15.13
	5.60	1.71	4.02	8.00	10.33	4.73	15.06
Bentazone	1.44	2.21	4.47	8.83	8.59	4.73	13.32
	2.88	2.25	4.10	9.14	8.87	4.52	13.39
	5.76	2.00	4.41	9.20	8.33	4.58	12.91
Unweeded		2.66	4.24	10.38	10.74	4.82	15.56
Hand-weeded		2.61	5.64	11.78	10.36	5.49	15.85
S.E. (D.F. = 21)		0.146	0.242	0.530	1.111	1.176	2.059

Table 4

Effect of different treatments on yield and yield components of pea

Treatment	Rate (kg/ha)	No. of pods/plant	Mean pod length (cm)	No. of seeds/pod	1000-seed wt (g)	Seed yield (t/ha)
Terbutryn/terbuthylazine	1.40	10.72	6.53	5.60	211	3.49
	2.80	8.35	6.27	5.30	204	3.21
	5.60	9.62	6.26	5.17	209	3.13
Bentazone	1.44	10.40	6.28	5.72	204	3.57
	2.88	11.25	6.25	5.10	201	3.35
	5.76	9.92	6.36	5.82	204	3.30
Unweeded		8.67	6.07	5.60	203	3.37
Hand-weeded		11.95	6.38	5.52	219	3.77
S.E. (D.F. = 21)		1.607	0.180	0.241	8.9	0.532

Table 5

Effect of various treatments on nitrogen content in seed, straw and whole plant of pea at maturity

Treatment	Rate (kg/ha)	N (mg/plant)		
		Seed	Straw	Total
Terbutryn/terbuthylazine	1.40	411	39	450
	2.80	285	33	318
	5.60	350	31	381
Bentazone	1.44	357	34	391
	2.88	375	35	410
	5.76	357	31	388
Unweeded		293	32	325
Hand-weeded		443	39	482
S.E. (D.F. = 21)		67.2	5.6	71.3

Discussion

Effects of herbicides

In the present study, conducted in the field, the number of nodules/plant (Fig. 2), nodule dry weight/plant (Fig. 3) and nitrogenase activity (Fig. 1) were not influenced significantly ($P>0.05$) by various treatments at most of the observation periods. It is possible that, despite the large differences in these parameters, the treatments did not differ significantly due to large variability in the data. Large variability in these parameters is not uncommon. Coefficients of variation of 40% for number of nodules (van Rensburg and Strijdom, 1984), 33–37% for nodule weight (van Rensburg and Strijdom, 1984; Jessen et al., 1987) and 41–87% for nitrogenase activity (Glenister and LaRue, 1986; Jessen et al., 1987) have been reported in the literature. Other workers have also observed very large differences between treatments in number and weight of nodules (Khokhar and Malik, 1988) and nitrogenase activity (Igual et al., 1997), but the differences were nevertheless non-significant, probably due to high variability in the data. In the present studies the seeds were not inoculated with rhizobia and the native population of rhizobia in the field may not have been uniform. Inoculation of seeds might have helped to decrease the variability in the data.

Terbutryn/terbuthylazine had a more damaging effect than bentazone, while both led to fewer nodules and lower nodule dry weight than the hand-weeded treatment at all sampling times (Figs. 2 and 3). Nitrogenase activity tended to decrease at higher rates of herbicide application (Fig. 1). Kumar et al. (1981) reported poor nodulation in chickpea with 1.6 and 3.2 kg/ha each of simazine and prometryn. Simazine at 1.38 kg/ha markedly decreased the specific nitrogenase activity in lupins (De Felipe et al., 1987), while Kumar et al. (1981) could not detect nitrogenase activity in simazine-treated (at 1.6 and 3.2 kg/ha) chickpea plants, as leghaemoglobin did not develop at all in the nodules. Bentazone is known to decrease specific nitrogenase activity in kidney bean (Bethlenfalvay et al., 1979; Schnelle and Hensley, 1990) and soybean (Ozair and Moshier, 1988; Yueh and Hensley, 1993). Lower nodulation and nitrogenase activity in herbicide-treated plants could be due to poor shoot growth (Table 3). Poor shoot growth could be the result of decreased photosynthesis, as both the herbicides used in these studies are known to adversely affect photosynthesis in plants (Worthing et al., 1982; Dodge, 1990; Tomlin, 1995).

The highest nitrogenase activity in the hand-weeded treatment was observed 8 weeks after sowing. As the plant age increased the nitrogenase activity decreased. The period 12 weeks after sowing corresponded with the seed-filling stage. Generally nitrogenase activity is higher at the flowering stage and decreases at seed-filling (Herridge and Pate, 1977; Pate and Herridge, 1978; Minchin et al., 1980; Jensen, 1987).

Seed yield was lower in herbicide treatments as compared to the hand-weeded treatment (Table 4), probably as a result of the effect of herbicides on plant growth in bentazone treatments and due both to the effect on plant growth and to the poor plant stand in terbutryn/terbuthylazine treatments, as terbutryn/terbuthylazine at higher rates killed some plants. This may be due to enhanced uptake by the plants owing to the sufficient soil moisture available following rainfall (data not presented).

The pre-emergence application of terbutryn/terbuthylazine at all rates of application was very effective against all weeds (Table 1), probably not only due to its strong herbicide effect but also due to the greater soil moisture available at the time of application due to rainfall. Bentazone did not control *Polygonum aviculare, Poa annua* or *Elymus repens,* as it is not so effective against these weeds (BASF, 1996).

Effects of weeds

Plants in the hand-weeded treatment had higher total nitrogenase activity than the unweeded control 8 and 10 weeks after sowing, whereas the unweeded treatment had higher nitrogenase activity than the hand-weeded treatment 7 and

12 weeks after sowing (Fig. 1). Sandhu et al. (1991) observed higher specific nitrogenase activity in a hand-weeded treatment over an unweeded control in inoculated lentils and they were of the view that this might have resulted from the better availability of space, nutrients, water and solar energy due to the removal of weeds. However, in lupins De Felipe et al. (1987) and Pozuelo et al. (1989) found higher specific nitrogenase activity in weedy plots and they were of the view that the greater activity observed in non-inoculated plants may be due to less nitrate being available to lupins as a consequence of competition for nutrients. Brockwell et al. (1995) also supported this view. In the present studies the seed was not inoculated with rhizobia, as inoculation is not recommended for peas in the United Kingdom (Gane et al., 1984) due to the presence of sufficient pea rhizobia in the soils. Thus, the plants were dependent for nodulation on the native strains of *Rhizobium* in the soil. The higher nitrogenase activity found at some sampling dates in the unweeded treatment compared to the hand-weeded treatment might be due to the reasons proposed by De Felipe et al. (1987) and Brockwell et al. (1995).

Weeds reduced seed yield by 11% (Table 4). The lower yields observed in the unweeded as compared to the hand-weeded treatment were probably due to crop–weed competition for nutrients, light and space (De Felipe et al., 1987; Kumar and Kolar, 1989; Sandhu et al., 1991), rather than competition for moisture, as the crop received enough rainfall during the whole growing season.

Possible alternatives to nitrogenase activity data

The nitrogen content in the plants, determined at maturity, did not correlate significantly (r = 0.019, 0.461, 0.620 and 0.073 at 7, 8, 10 and 12 weeks after sowing, respectively) with the nitrogenase activity measured at different periods during crop growth. Similarly the nitrogenase activity did not correlate well with shoot dry weight at different periods of measurement (r = 0.576, 0.656, 0.352 and 0.127 at 6, 8, 10 and 12 weeks after sowing, respectively).

Witty and Minchin (1988) and Minchin et al. (1994) suggested that a closed acetylene reduction assay should not be used for measuring nitrogenase activity due to the errors (plant disturbance and acetylene-induced decline) associated with this technique. They suggested the use of simple alternative measurements such as dry weight, yield and total nitrogen. Relationships between plant-N content and straw, seed and total above-ground plant biomass are shown in Fig. 4. The nitrogen content (mg/plant) in plants was positively and significantly ($p < 0.05$) correlated with seed (r = 0.987) and straw (r = 0.939) yields per plant. Other researchers also believe that plant-N and plant biomass are normally well correlated (Minchin et al., 1986; Witty and Minchin, 1988; Mytton, 1988). These strong relationships between plant biomass and plant-N content suggest that researchers may depend on these parameters for studying the effects of treatments on nitrogen fixation rather than measuring nitrogenase activity.

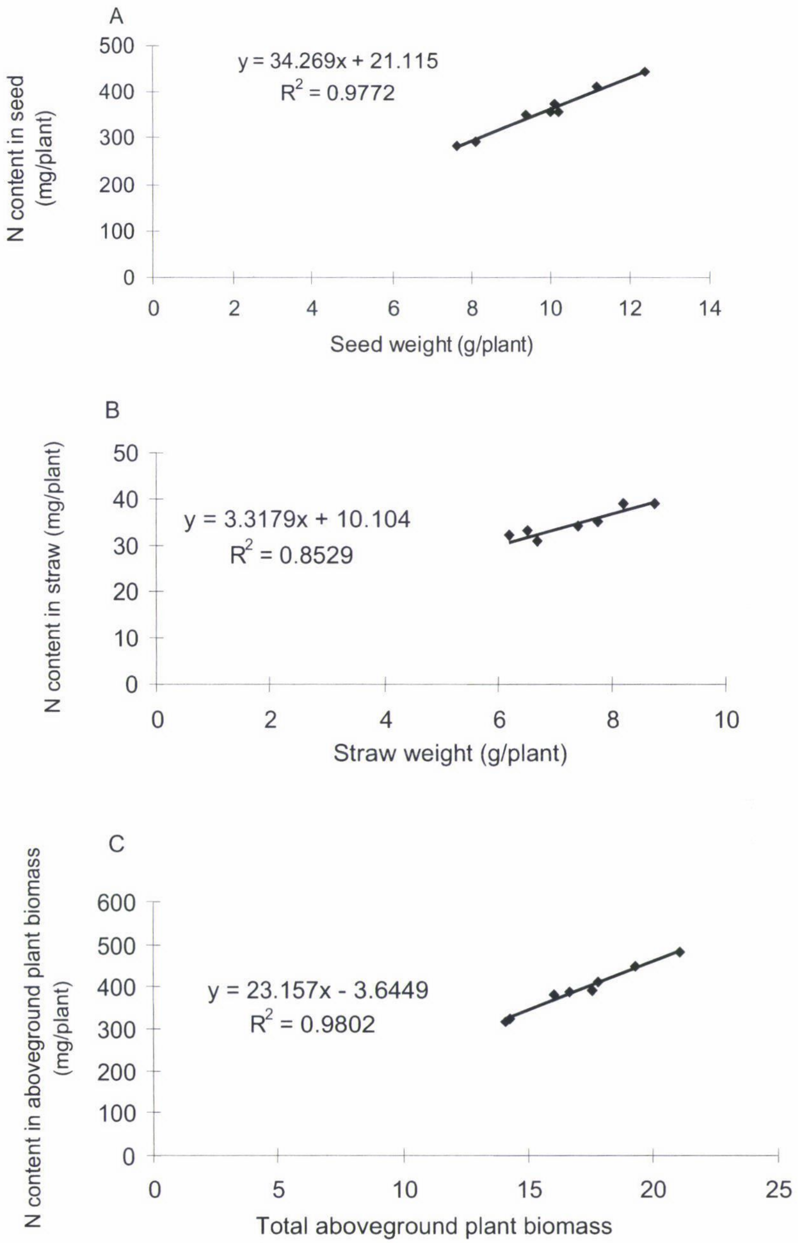

Fig. 4. Relationship between nitrogen content per plant and (A) seed weight per plant, (B) straw weight per plant and (C) total aboveground plant biomass per plant at maturity in peas

Lower nitrogen content/plant was found in herbicide-treated plants compared to the hand-weeded ones (Table 5). This, however, may not always happen, as plants can also take nitrogen from the soil. Hence in some situations, if the effect of the given treatment on nitrogen fixation or plant growth is low, the plants can compensate by nitrogen uptake from the soil (Rennie et al., 1982; Rennie and Dubetz,1984a, b).

In the words of Witty and Minchin (1988) "despite progress in understanding many areas of biological nitrogen fixation, its accurate measurement in the field remains a mystery". Investigations on plant weight, yield or total nitrogen content could give some indication of the effect (negative or positive) of any treatment on nitrogen fixation and, though not a complete solution, could be one step towards understanding the mystery.

Practical problems, possible solutions and future direction of research

The results showed that to obtain higher yields of pea it is necessary to control weeds. Weeds can be effectively controlled using pre- or post-emergence herbicides. However, some environmental conditions, such as a dry seed bed, may lower the efficiency of pre-emergence herbicides, while too much moisture, possibly due to irrigation or rainfall, may cause herbicides to have adverse effects on the crop at higher rates. Herbicides may decrease nodulation and nitrogenase activity, so it is essential to screen more herbicides and use only those which can control weeds effectively without adversely affecting nitrogen fixation in legumes. Herbicides may decrease nodulation and nitrogenase activity not only by influencing plant growth but also by affecting rhizobia. Though the herbicides used in the present studies did not adversely affect the growth of rhizobia *in vitro* at the recommended field application rates (Singh and Wright, 2002), other herbicides need to be tested for their possible adverse effects on rhizobia.

Weeds may sometimes enhance nitrogenase activity in legumes by decreasing the nitrate in the soil, but this is not a real benefit as weeds may rob nutrients, compete with the crop plants for other resources and thus decrease crop yields. In the present studies weeds decreased pea seed yield by 11%. Nutrient uptake, including nitrogen, by weeds results in a loss of soil fertility and must thus be checked. The application of herbicides must be done at the correct rate, as incorrect rates of application or the overlapping of sprayed areas may result in adverse effects on crop growth and yield. Some herbicides may have long persistence in the soil and, if applied to one crop, may show an adverse effect on biological nitrogen fixation in the succeeding legume crop in the rotation.

Large variabilities in nodulation and nitrogenase activity are often found in field experiments. It is possible that the inoculation of seed with rhizobia might decrease this variability. As a flow-through gas system cannot be used for measuring nitrogenase activity in field experiments (Vessey, 1994) and plant disturbance also decreases nitrogenase activity (Minchin et al., 1986, 1994; Witty and Minchin 1988) better and cheaper methods need to be developed for measuring biological nitrogen fixation under field conditions.

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References

AOAC (1955): *Official Methods of Analysis*. Association of Official Agricultural Chemists, Washington DC, USA.

BASF (1996): *Product Manual*. BASF plc Agricultural Division, Cheadle, UK.

Bethlenfalvay, G. J., Norris, R. F., Phillips, D. A. (1979): Effect of bentazon, a Hill reaction inhibitor, on symbiotic nitrogen-fixing capability and apparent photosynthesis. *Plant Physiol.,* **63**, 213–215.

Brockwell, J., Bottomley, P. J., Thies, J. E. (1995): Manipulation of rhizobia microflora for improving legume productivity and soil fertility: A critical assessment. *Plant Soil,* **174**, 143–180.

De Felipe, M. R., Fernandez-Pascual, M., Pozuelo, J. M. (1987): Effects of the herbicides lindex and simazine on chloroplast and nodule development, nodule activity, and grain yield in *Lupinus albus* L. *Plant Soil,* **101**, 99–105.

Dodge, A. D. (1990): The mode of action and metabolism of herbicides. pp. 201–215. In: Hance, R. J., Holly, K. (eds.), *Weed Control Handbook: Principles,* 8th Edition. Blackwell Scientific Publications, Oxford, UK.

Gane, A. J., Biddle, A. J., Knott, C. M., Eagle, D. J. (1984): *The Pea Growing Handbook.* Processors and Growers Research Organisation, Thornhaugh, UK.

Glenister, R., LaRue, T. A. (1986): Non-destructive estimate of nitrogenase activity (C_2H_2) of field-grown soybean. *Plant Soil,* **96**, 137–140.

Hardy, R. W. F., Burns, R. C., Holsten, R. D. (1973): Applications of the acetylene-ethylene assay for measurement of nitrogen fixation. *Soil Biol. Biochem.,* **5**, 47–81.

Herridge, D. F., Pate, J. S. (1977): Utilization of net photosynthate for nitrogen fixation and protein production in an annual legume. *Plant Physiol.,* **60**, 759–764.

Igual, J. M., Rodríguez-Barrueco, C., Cervantes, E. (1997): The effects of aluminium on nodulation and symbiotic nitrogen fixation in *Casuarina cunninghamiana* Miq. *Plant Soil,* **190**, 41–46.

Jensen, E. S. (1987): Seasonal patterns of growth and nitrogen fixation in field-grown pea. *Plant Soil,* **101**, 29–37.

Jessen, D. L., Barnes, D. K., Vance, C. P., Heichel, G. H. (1987): Variation for activity of nodule nitrogen and carbon assimilating enzymes in alfalfa. *Crop Sci.,* **27**, 627–631.

Jonsson, K. (1988): *Instructions for Measurements of Nitrogenase Activity by the Acetylene Reduction Method.* Stencil No.7. Department of Forest Site Research, Swedish University of Agricultural Sciences, Umeå, Sweden.

Khokhar, S. N., Malik, B. A. (1988): Effect of herbicides on nodulation and nitrogenase activity of chickpea. *Pakistan J. Agric. Res.,* **9**, 493–497.

Knott, C. M. (1987): A key for stages of development of the pea (*Pisum sativum*). *Annals Appl. Biol.,* **111**, 233–244.

Kumar, K., Kolar, J. S. (1989): Effect of chemical weed control and *Rhizobium* inoculation on the yield of lentil. *J. Res. Punjab Agric. Univ.,* **26**, 19–24.

Kumar, S., Pahwa, S. K., Promila, K., Sharma, H. R. (1981): Effect of simazine and prometryne on the growth and nodulation of chickpea (*Cicer arietinum* L.). *J. Agric. Sci. (Camb.),* **97**, 663–668.

Masterson, C. L., Murphy, P. M. (1980): The acetylene reduction technique. pp. 8–33. In: Subba Rao, N. S. (ed.), *Recent Advances in Biological Nitrogen Fixation.* Oxford and IBH Publishing Company, New Delhi, India.

Minchin, F. R., Sheehy, J. E., Witty, J. F. (1986): Further errors in the acetylene reduction assay: Effects of plant disturbance. *J. Exper. Bot.,* **37**, 1581–1591.

Minchin, F. R., Summerfield, R. J., Neves, M. C. P. (1980): Carbon metabolism, nitrogen assimilation and seed yield of cowpea (*Vigna unguiculata* L. Walp.) grown in an adverse temperature regime. *J. Exper. Bot.,* **31**, 1327–1345.

Minchin, F. R., Witty, J. F., Mytton, L. R. (1994): Reply to 'Measurement of nitrogenase activity in legume root nodules: In defense of the acetylene reduction assay' by J. K. Vessey. *Plant Soil,* **158,** 163–167.

Minchin, F. R., Witty, J. F., Sheehy, J. E., Müller, M. (1983): A major error in the acetylene reduction assay: Decreases in nodular nitrogenase activity under assay conditions. *J. Exper. Bot.,* **34**, 641–649.

Mytton, L. R. (1988): Workshop synthesis and recommendations developed from group discussions. pp. 373–379. In: Beck, D. P., Materon, L. A. (eds.), *Nitrogen Fixation by Legumes in Mediterranean Agriculture.* Martinus Nijhoff, Dordrecht, the Netherlands.

Ozair, C. A., Moshier, L. J. (1988): Effect of postemergence herbicides on nodulation and nitrogen fixation in soybeans (*Glycine max*). *Appl. Agric. Res.,* **3**, 214–219.

Pate, J. S., Herridge, D. F. (1978): Partitioning and utilization of net photosynthate in a nodulated annual legume. *J. Exper. Bot.,* **29**, 401–412.

PGRO (1993): *Pea Leaf Wax Assessment.* Information Sheet Number 143. Processors and Growers Research Organisation, Thornhaugh, UK.

Pozuelo, J. M., Fernandez-Pascual, M., Lucas, M. M., De Felipe, M. R. (1989): Effect of eight herbicides from five different chemical groups on nitrogen fixation and grain yield in *Lupinus albus* L. grown in semi-arid zones. *Weed Res.,* **29**, 419–425.

Rennie, R. J., Dubetz, S. (1984a): Effect of fungicides and herbicides on nodulation and N_2 fixation in soybean fields lacking indigenous *Rhizobium japonicum. Agron. J.,* **76**, 451–454.

Rennie, R. J., Dubetz, S. (1984b): Multistrain vs. single strain *Rhizobium japonicum* inoculants for early maturing (00 and 000) soybean cultivars: N_2 fixation quantified by ^{15}N isotope dilution. *Agron. J.,* **76**, 498–502.

Rennie, R. J., Dubetz, S., Bole, J. B., Muendel, H. H. (1982): Dinitrogen fixation measured by ^{15}N isotope dilution in two Canadian soybean cultivars. *Agron. J.,* **74**, 725–730.

Sandhu, P. S., Dhingra, K. K., Bhandari, S. C., Gupta, R. P. (1991): Effect of hand-hoeing and application of herbicides on nodulation, nodule activity and grain yield of *Lens culinaris* Med. *Plant Soil,* **135**, 293–296.

Schnelle, M. A., Hensley, D. L. (1990): Effects of pesticides upon nitrogen fixation and nodulation by dry bean. *Pesticide Sci.,* **28**, 83–88.

Singh, G., Wright, D. (1999): Effects of herbicides on nodulation, symbiotic nitrogen fixation, growth and yield of pea (*Pisum sativum*). *J. Agric. Sci. (Camb.),* **133**, 21–30.

Singh, G., Wright, D. (2002): *In vitro* studies on the effects of herbicides on the growth of rhizobia. *Letters Appl. Microbiol.,* **35**, 12–16.

Tomlin, C. (ed.) (1995): *The Pesticide Manual,* 10th Edition. British Crop Protection Council, Farnham and Royal Society of Chemistry, Cambridge, UK.

Turner, G. L., Gibson, A. H. (1980): Measurement of nitrogen fixation by indirect means. pp. 111–138. In: Bergersen, F. J. (ed.), *Methods for Evaluating Biological Nitrogen Fixation.* John Wiley and Sons Ltd., London, UK.

van Rensburg, H. J., Strijdom, B. W. (1984): Effect of herbicides on survival of rhizobia and nodulation of peas, groundnuts and lucerne. *South African J. Plant Soil,* **1**, 135–138.

Vessey, J. K. (1994): Measurement of nitrogenase activity in legume root nodules: In defense of the acetylene reduction assay. *Plant Soil,* **158**, 151–162.

Witty, J. F., Minchin, F. R. (1988): Measurement of nitrogen fixation by the acetylene reduction assay: Myths and mysteries. pp. 331–344. In: Beck, D. P., Materon, L. A. (eds.), *Nitrogen Fixation by Legumes in Mediterranean Agriculture.* Martinus Nijhoff, Dordrecht, the Netherlands.

Worthing, C. R., Richardson, W. G., Taylor, W. A. (1982): Properties of herbicides. pp. 106-157. In: Roberts, H. A. (ed.), *Weed Control Handbook: Principles,* 7th Edition. Blackwell Scientific Publications, London, UK.

Yueh, L. Y., Hensley, D. L. (1993): Pesticide effect on acetylene reduction and nodulation by soybean and lima bean. *J. American Soc. Hort. Sci.,* **118**, 73–76.

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MOVEMENT OF NITROGEN IN A SANDY LOAM SOIL UNDER A CONTINUOUS MAIZE–WHEAT CROPPING SYSTEM

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Nitrogen (N) movement in the soil resulting from the long-term application of fertilizer N is an environmental concern when it reaches the groundwater. The distribution of N in the profile of an alkaline sandy loam soil (Typic Haplustept) and its relationship with N uptake by plants was studied after 22 years of continuous cultivation in an annual crop rotation involving maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.). Soil samples were collected to a depth of 1.2 m from the 0–0.15, 0.15–0.30, 0.30–0.45, 0.45–0.60, 0.60–0.90 and 0.90–1.20 m layers and analysed for alkaline $KMnO_4$-oxidisable N (available N) and mineral N (NH_4-N and NO_3-N). The continuous addition of increasing levels of N resulted in an increase in N content, whereas the combined application of N, P and K caused a decline in its availability. Mineral N (2 M KCl-extractable NH_4-N and NO_3-N) was the lowest in the $N_{120}P_{35}K_{33.2}$ treatment plot. The available N and NH_4-N decreased with increasing soil depth. However, variations in NO_3-N concentration due to differential rates of fertilizer application were observed only to a depth of 0.45 m. This effect was more pronounced in the $N_{180}P_{17.5}K_{33.2}$ plot. Regression equations were used to predict N uptake by wheat using the N status in different soil layers as independent variables. Multiple regression analysis indicated that the predictability of the relationship between N uptake and available N improved considerably when its status to a soil depth of 0.45 m was included. In the case of NH_4-N, a noticeable increase in the coefficient of determination (R^2) occurred to a depth of 0.90 m. The R^2 value of NO_3-N with the N uptake by wheat was quite low in the top layers (to a depth of 0.30 m). However, an increase in the R^2 value was observed when lower depths (beyond 0.30 m) were included in the regression analysis, suggesting that the inclusion of subsoil N status is important to achieve better and profitable N supply systems in crop production.

Key words: soil depth, available and mineral N, fertilizer addition, N uptake

Introduction

Nitrogen (N) is a major plant nutrient required for high yields of most agricultural crops. However, the management of nitrogenous fertilizer for crop production remains a difficult task due to the numerous transformation and loss mechanisms, such as NH_3 volatilization, nitrification following denitrification, chemical and microbial fixation, leaching and runoff (Hussain et al., 2003). Even N applied on the soil surface becomes distributed within the soil profile with subsequent irrigations (Asadi et al., 2002). The nitrate form of N (NO_3-N) is water-soluble and susceptible to transport to the groundwater, causing the degradation of aquifer water quality in the soils of the Indo-Gangetic plains (Singh et al., 1995a). The rise in the NO_3-N concentration in the groundwater of Punjab with the increased use of fertilizer was also reported by Singh et al. (1991). On the other hand, some part of the ammonium form of N is adsorbed on soil particles; consequently, this form of N is not subject to so much movement as nitrate-N (Sharma et al., 1985). This obviously indicates the need to measure the N fertility status of a soil periodically in order to study the environment and economy issues involved in N fertilizer use. The roots of field crops generally penetrate the soil to a considerable depth. The crops absorb nutrients from sub-surface soil layers depending on the root morphology. The contribution of nutrients in sub-surface soil layers towards plant nutrition has also been reported by Kapur et al. (1988) and Setia and Sharma (2004). There is a need to study the influence of differential rates of fertilizer addition over a period of time in a continuous cropping system on the depth-wise distribution of N forms and its relationship with plant N uptake. The present study was planned to study the effect of N, P and K application on the movement of N as a function of N uptake after 22 cycles of crop rotation (maize–wheat) in a long-term fertilizer experiment.

Materials and methods

Site and soil description

Eleven different treatments were selected from a long-term fertilizer experiment with a maize–wheat cropping system initiated at the Punjab Agricultural University Farm, Ludhiana (30° 56' N latitude and 75°27' E longitude) on a sandy loam soil (Typic Haplustept) in the summer season of 1979. The experimental area is characterized as semi-arid sub-tropical. The mean minimum temperature ranged from 6°C during December–January to 26.2°C during June and the maximum temperature from 17.8°C during January to 40°C in June. The average annual rainfall was 760 mm. More than 80% of the total rainfall is received from July to September in the monsoon season.

In the maize–wheat rotation, wheat was sown each year in the first week of November in rows 22.5 cm apart and was irrigated to a depth of 7.5 cm three to four times during the growing season. It was harvested in the first fortnight of April. Hand weeding was done and standard pest control measures were followed. After harvesting wheat, the field was kept fallow up to the last week of May when pre-sowing irrigation (10 cm) and land preparation for the maize crop started. Maize was sown in mid-June of every year at a row × plant spacing of 60 × 22 cm. The fertilizer treatment comprised combinations of four levels of N (0, 60, 120 and 180 kg N ha^{-1}), three levels of P (0, 17.5 and 35 kg P ha^{-1}) and two levels of K (0 and 33.2 kg K ha^{-1}) in a $3^2 \times 2$ partially factorial randomized block design. Each treatment was replicated four times. The sources of N, P and K were urea, single superphosphate and muriate of potash, respectively. The N, P and K were applied to both the crops, whereas Zn, as 40 kg $ZnSO_4$ ha^{-1}, was applied to maize only.

At the start of the experiment, the experimental soil (0–0.15 m) had pH 8.2 (1:2 soil water suspension) and electrical conductivity 0.132 dS m^{-1} (in the supernatant of a 1:2 soil:water solution stirred well and kept overnight). The soil contained 3.45 g kg^{-1} organic carbon. The initial contents of alkaline $KMnO_4$-oxidisable N (Subbiah and Asija, 1956), 0.5 M $NaHCO_3$ (pH 8.5)-extractable P (Olsen et al., 1954), 1 N NH_4OAc (pH 7.0)-extractable K (Jackson, 1967) and 0.15% $CaCl_2$-extractable S (Williams and Steinberg, 1959) were 52.2, 6.5, 56.7 and 16.2 mg kg^{-1}, respectively.

Treatments and laboratory analysis

Depth-wise soil samples (0–0.15, 0.15–0.30, 0.30–0.45, 0.45–0.60, 0.60–0.90 and 0.90–1.20 m) were collected from the selected treatments after the harvesting of wheat (44th crop in the sequence) during the winter season of 2000–2001. The treatments selected for the present study were $N_0P_0K_0$, $N_{120}P_0K_0$, $N_{180}P_0K_0$, $N_{120}P_{17.5}K_0$, $N_{120}P_{35}K_0$, $N_{180}P_{17.5}K_0$, $N_{180}P_{35}K_0$, $N_{120}P_{17.5}K_{33.2}$, $N_{120}P_{35}K_{33.2}$, $N_{180}P_{17.5}K_{33.2}$ and $N_{180}P_{35}K_{33.2}$. The physico-chemical properties of the entire soil profile (0–1.20 m) of the control plot after the harvesting of wheat (44th crop) are given in Table 1. These samples were analysed for available N by the alkaline permanganate method as modified by Subbiah and Asija (1956). NH_4-N and NO_3-N were determined by extracting the soil with 2M KCl (1:10 soil – extractant ratio). The filtrate was distilled with MgO + Devarda's alloy to determine NH_4-N and NO_3-N by absorbing ammonia in boric acid (Bremner, 1965).

Statistical analysis

The relationship between applied fertilizer and forms of N was computed by linear regression analysis. Stepwise multiple regression analysis was used to compute the relationship between N uptake and forms of N in different soil layers (0–1.20 m) (Panse and Sukhatme, 1967).

Results and discussion

Alkaline $KMnO_4$-oxidisable N

The alkaline $KMnO_4$-oxidisable N (available N) content ranged from 25.2 to 47.6 mg kg^{-1} in the 0–0.15 m depth after 22 cycles of continuous cropping and fertilization, and it decreased with increasing soil depth (Fig. 1). Among the various treatments, the available N content in the soil increased with increasing levels of N. This increase may be attributed to the accumulation of N from the applied fertilizer. The combined application of N and P or N, P and K resulted in a decline in the available N status of the soil. The yield of wheat (grain and straw) was higher in plots receiving N, P and K than in plots fertilized with N and P or N alone (Anonymous, 2001). This may have resulted in the higher removal of N from both the applied and native N reserves, causing a decline in the available N pool of the soil. Such behaviour was more pronounced in plots receiving higher fertilization rates. These results are consistent with the findings of Singh et al. (1995b). The N+P treatments tended to show a lower N concentration than the corresponding N-only treatments at comparable soil depth and the differences in N concentration between the N+P and the corresponding N-only treatments increased with the P rate (Fig. 1). A decline in available N with increasing soil depth may be attributed to the extractant used for this estimation, since the alkaline permanganate method extracts soil N both from inorganic N and from the organically bound N fraction. More than 95% of soil N is organic in nature. The leaching of the organic fraction of soil N is negligible. A decrease in available N with increasing soil depth was observed. Holford (1981) and Brar and Pasricha (1998) also reported similar results.

Table 1

Characteristics of various soil depth increments for control plot after 22 cycles of continuous maize–wheat sequence

Property	Depth (m)					
	0–0.15	0.15–0.30	0.30–0.45	0.45–0.60	0.60–0.90	0.90–1.20
pH (1 : 2 soil : water)	7.35	7.58	7.52	7.85	7.88	7.96
EC (dS m^{-1})	0.24	0.18	0.15	0.13	0.18	0.15
Organic carbon (g kg^{-1})	3.00	2.56	2.74	2.01	1.42	0.92
$CaCO_3$ equivalent (%)	nil	nil	nil	nil	nil	nil

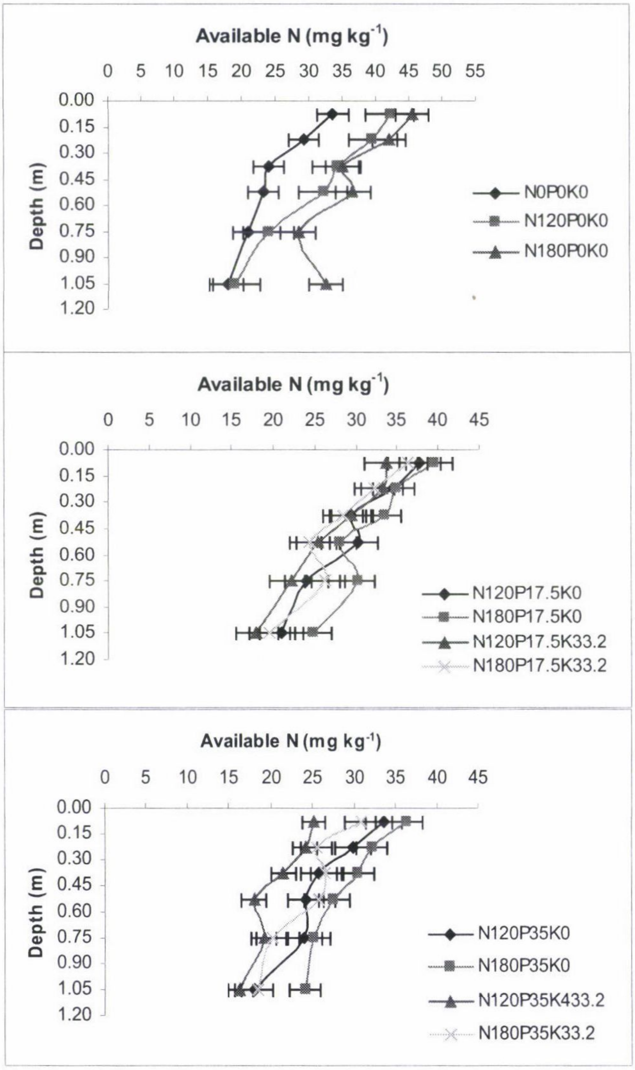

Fig. 1. Soil-available N at various soil depth increments after 22 cycles of a maize–wheat sequence. Horizontal bars indicate standard errors

Mineral N (NH_4-N + NO_3-N)

The data in Figure 2 indicated a decline in NH_4-N content with increasing soil depth, irrespective of the fertilizer treatment. However, an increasing level of N resulted in a rise in the NH_4-N content of the soil. The NH_4-N content at different soil depths in plots receiving N and P or N, P and K was much lower than in plots receiving N alone. This can be ascribed to higher N removal by crops in the NP- and NPK-treated plots (Anonymous, 2001). Moreover, the applied N might have moved to lower depths (within the rooting zone) and have been utilized more efficiently by the roots in the treatments yielding higher than the N-treated plots. The NH_4-N content is higher in surface soils due to its adsorption on organic and inorganic colloidal complexes (Walia et al., 1998). The organic carbon was higher in the surface layer because more roots and plant residues are left in the surface soil. Therefore, a greater amount of NH_4-N from unutilized N fertilizer may have been adsorbed on the exchange complex of the surface layer (0–0.15 m). These results corroborate the findings of Gupta et al. (1989).

The concentration of NO_3-N increased with increasing depth to 0.45 m, beyond which this effect was not consistent (Fig. 3). This may be attributed to the mineralization of organic N and the microbial transformation of the inorganic form of N in the upper soil layers. The NO_3-N concentration was higher at all depths in the N-treated plots as compared with unfertilized plots even after 22 years of cropping. The NO_3-N ions are not adsorbed on an exchangeable complex and move freely with percolating water, leading to an increase in the NO_3-N concentration in the lower soil depths. Sharma et al. (1985) and Gupta et al. (2000) also reported the movement of fertilizer N in the form of NO_3-N. The accumulation of NO_3-N at lower soil depths can be attributed to the movement of higher concentrations of applied N along with percolating water, as the experimental soil is sandy loam and the proportion of smaller pores is comparatively low, increasing the mobility of NO_3 ions in the soil with a given amount of fertilizer (Munson and Nelson, 1963; Sharma and Singh, 1982).

Among the two inorganic N forms, the concentration of NH_4-N was higher than that of NO_3-N in all the treatment plots. This may be due to the oxidized conditions that prevail during wheat growth, resulting in the acceleration of the nitrification process. This might have caused the conversion of a sizeable amount of NH_4-N to NO_3-N for rapid utilization by the wheat plants. At the same time, the water applied during crop growth also facilitated the movement of NO_3-N to deeper soil layers.

Distribution of forms of N in the soil profile (0–1.20 m) after the wheat harvest

The distribution of available N, NH_4-N and NO_3-N in the soil profile was higher in N-treated plots than in the control or in NP- and NPK-treated plots (Table 2). The lower amount of N in the NP or NPK plots may be ascribed to the

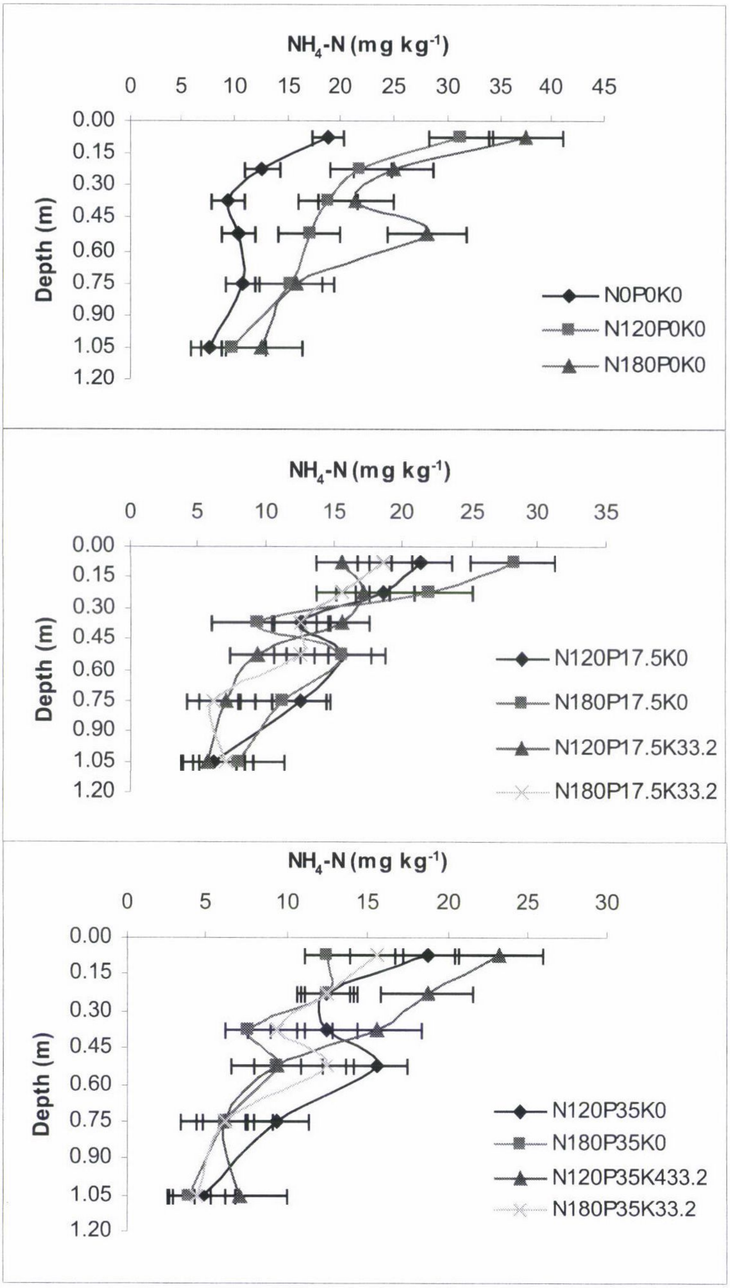

Fig. 2. Soil NH_4-N at various soil depth increments after 22 cycles of a maize–wheat sequence. Horizontal bars indicate standard errors

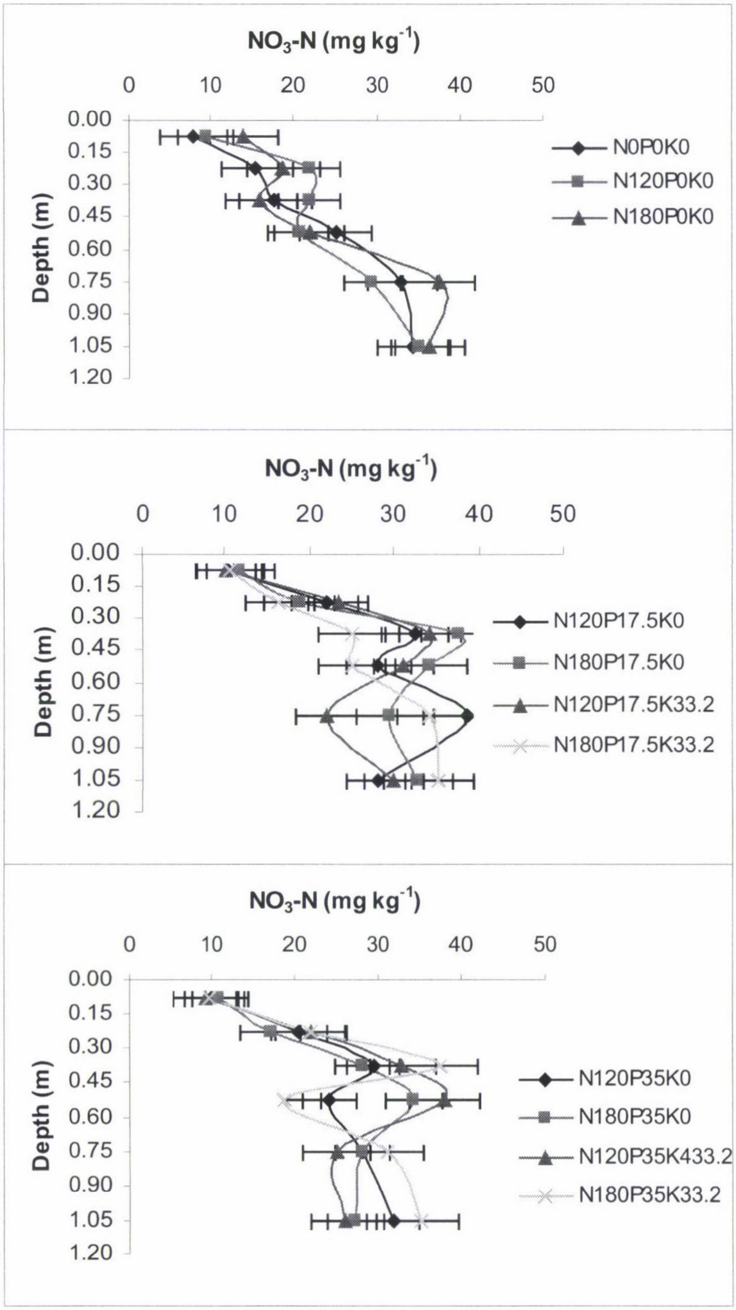

Fig. 3. Soil NO_3-N at various soil depth increments after 22 cycles of a maize–wheat sequence. Horizontal bars indicate standard errors

Table 2

Distribution of N (kg ha^{-1}) in the soil profile (0–1.20 m) after the wheat harvest

Treatment	Available N	NH_4-N	NO_3-N
$N_0P_0K_0$	113	130	402
$N_{120}P_0K_0$	140	278	406
$N_{180}P_0K_0$	168	337	477
$N_{120}P_{17.5}K_0$	133	212	461
$N_{180}P_{17.5}K_0$	146	227	494
$N_{120}P_{35}K_0$	120	176	449
$N_{180}P_{35}K_0$	135	188	403
$N_{120}P_{17.5}K_{33.2}$	120	123	406
$N_{180}P_{17.5}K_{33.2}$	128	172	433
$N_{120}P_{35}K_{33.2}$	96	125	432
$N_{180}P_{35}K_{33.2}$	112	143	442

higher N requirements of crops in plots receiving NP or NPK application than in plots receiving N fertilizer alone. The data in Table 1 indicated a low N content in the control plot. Probably, mineralizable organic N decreased to a larger extent in this plot due to continuous cropping without fertilizer application. The distribution of NO_3-N in the soil profile was higher than that of NH_4-N because maize, which preceded wheat in the sequence, is a rainy season crop and part of the applied N might have leached down to deeper soil layers as the rainy season progressed. Therefore, the residual effect of applied N on the following crop cannot be ignored. These observations suggest the need for the regular monitoring of mineral N in the soil profile, particularly in fields where a continuous cropping system is followed over a number of years, so as to mine the residual soil N by introducing a cut in the fertilizer N rate of the crop to follow.

The relationship between applied fertilizer and forms of N was computed by simple linear regression analysis (Table 3), which indicated that the application of different doses of N fertilizer had a significant ($p<0.05$) effect on available N as well as on mineral N. In all cases the effect of P and K fertilizer was negative (though not significant), but a significant ($p<0.05$) negative effect on available N and NH_4-N was found with K fertilization, which may be ascribed to the increased uptake of N with increasing levels of K addition. It should be noted that the yield of both the crops in the sequence increased with the addition of NP or NPK. Obviously more N was absorbed in these treatments to meet crop demand for N.

Table 3

Relationship between applied fertilizer and N in the soil profile using linear regression analysis (0–1.20 m)

	R square	N-coeff	P-coeff	K-coeff
Available N	0.946*	0.287	–0.354	–0.512*
NH_4-N	0.758*	0.882	–1.32	–1.23*
NO_3-N	0.261	0.321	–0.125	–0.202

*Significant at the 5% level

Table 4

Relationship between N uptake and forms of N in different soil layers

Equation	R^2 value
Available N	
X_1	0.14
$X_1 + X_2$	0.27
$X_1 + X_2 + X_3$	0.79
$X_1 + X_2 + X_3 + X_4$	0.88
$X_1 + X_2 + X_3 + X_4 + X_5$	0.90
$X_1 + X_2 + X_3 + X_4 + X_5 + X_6$	0.91
NH_4-N	
X_1	0.25
$X_1 + X_2$	0.34
$X_1 + X_2 + X_3$	0.35
$X_1 + X_2 + X_3 + X_4$	0.37
$X_1 + X_2 + X_3 + X_4 + X_5$	0.82
$X_1 + X_2 + X_3 + X_4 + X_5 + X_6$	0.98
NO_3-N	
X_1	0.03
$X_1 + X_2$	0.08
$X_1 + X_2 + X_3$	0.63
$X_1 + X_2 + X_3 + X_4$	0.63
$X_1 + X_2 + X_3 + X_4 + X_5$	0.64
$X_1 + X_2 + X_3 + X_4 + X_5 + X_6$	0.75

X_1, X_2, X_3 , X_4, X_5 , X_6 indicate 0–0.15, 0.15–0.30, 0.30–0.45, 0.45–0.60, 0.60–0.90 and 0.90–1.20 m soil depths, respectively

Relationship between N uptake and forms of N in different soil layers

The relationship between N uptake and soil N in different soil layers (0–1.20 m) was computed to find out the extent of contribution of N from lower soil layers towards N nutrition. The relative importance of different forms of N in the soil profile (0–1.20 m) for the uptake of N by wheat was computed by stepwise regression analysis (Table 4). In the surface layer (0–0.15 m), NH_4-N was the most important variable contributing to the N nutrition of wheat, as 25% variations could be accounted for by this variable. The R^2 value between N uptake and NH_4-N increased by 9% when the N status of the subsoil (0.15–0.30 m) was also taken into consideration. The inclusion of lower depths (0.30–0.45 and 0.45–0.60 m) did not improve the R^2 value, but increases of 45 and 16% with respect to NH_4-N were obtained by including the next soil depths (0.60–0.90 and 0.90–1.20 m) in the regression analysis. It is pertinent to mention here that N had been applied at 120 or 180 kg N ha^{-1} from the last 22 years, so the residual effect of applied N on the following crop cannot be ignored (Biswas and Benbi, 1997). The soil in the study was lighter and it is possible that N leaching took place. Wheat roots can penetrate as deep as 1.8 to 2 m (Gajri et al., 1989). Therefore, the plant is able to mine N from lower depths with advancing age (Kapur et al., 1988), resulting in a considerable change in predicted values when soil depths of 0.60–0.90 and 0.90–1.20 m were included. The inclusion of the available N status of the 0.30–0.45 m soil layer improved the R^2 value by 52%. Thus, the N present in the lower soil depths made a considerable contribution towards the N uptake by wheat, as the predictability increased with the inclusion of each successive lower soil depth in the regression analysis. The increase in the R^2 value by the inclusion of 0.30–0.45 m soil depth in the regression analysis for NO_3-N indicated the utility of taking the subsurface N status into consideration when recommending fertilizer N rates for crops.

Conclusions

The present study indicated that the continuous cropping of maize–wheat over a period of 22 years decreased all the N forms with increasing soil depth, irrespective of the fertilizer treatment. Available N and NH_4-N were the major fractions contributing to the N nutrition of wheat. The fertilization programme for any crop or cropping sequence should not be based merely on the N status of the surface layer alone. The inclusion of subsurface N status in fertilizer scheduling, particularly in light-textured soils, may help in the judicious use of fertilizer inputs.

References

Anonymous (2001): *Annual Reports of Project on Cropping System Research*. Department of Agronomy, Punjab Agriculture University, Ludhiana, India.

Asadi, M. E., Clemente, R. S., Gupta, A. D., Loof, R. N., Hansen, G. N. (2002): Impacts of irrigation on nitrate leaching and corn yield in acid sulphate soil in Thailand. *Agri. Water Mgt.*, **52**, 197–213.

Biswas, C. R., Benbi, D. K. (1997): N balance and N recovery after 22 years of maize-wheat-cowpea cropping in long term experiment. *Nutrient Cycling in Agroecosystem,* **47**, 107–114.

Brar, B. S., Pasricha, N. S. (1998): Long term use of organic and inorganic fertilizers in maize-wheat-cowpea cropping system on alluvial soils of Punjab. pp. 164–168 In: Swarup, A., Reddy, D., Prasad, R. N. (eds.), *Long Term Soil Fertility Management through Integrated Plant Nutrient Supply.* Proceedings of a National Workshop held during 2–4 April, 1998 at the Indian Institute of Soil Science, Bhopal, India.

Bremner, J. M. (1965): Nitrogen. pp. 595–624. In: Black, C. A. (ed.), *Methods of Soils Analysis. Part 2.* Agronomy Monograph No. **9**. American Society of Agronomy, Madison, Wisconsin, USA.

Gajri, P. R., Prihar, S. S., Arora, V. K. (1989): Effect of N and early irrigation on root development and water use by wheat on two soils. *Field Crops Res.,* **21**, 103–114.

Gupta, R. K., Dhillon, N. S., Dev, G. (1989): Profile distribution of various forms of soil N and their relationship with rice grain yield. *J. Indian Soc. Soil Sci.*, **37**, 174–176.

Gupta, R. K., Arora, B. R., Sharma, K. N. (2000): Effect of urea and manure addition on changes in mineral N content in soil profile at various growth stages of rice. *J. Indian Soc. Soil Sci.*, **48**, 787–792.

Holford, I. C. R. (1981): Change in N and organic carbon in wheat growing soils after grazed lucerne, extended fallowing and continuous wheat. *Australian J. Soil Res.*, **19**, 239–249

Hussain, P., Zia, M. S., Akhtar, M. E., Yasin, M. (2003): Nitrogen management and use efficiency with chlorophyll meter and leaf colour chart. *Pakistan J. Soil Sci.*, **22**, 1–10.

Jackson, M. L. (1967): *Soil Chemical Analysis*. Prentice Hall of India Pvt. Ltd., New Delhi, India.

Kapur, M. L., Sekhon, G. S., Singh, B. (1988): Contribution of surface and subsurface soil layers to the nutrition of pearl millet. *Indian J. Ecol.*, **15**, 43–47.

Munson, R. D., Nelson, W. L. (1963): Movement of applied N in soils. *J. Agri. Food Chemistry,* **11**, 193–201.

Olsen, S. R., Cole, C. V., Watanabe, F. S., Dean, L. A. (1954): *Estimation of available P in soils by extraction with* $NaHCO_3$. USDA Circ., **58**, 939.

Panse, V. G., Sukhatme, P. V. (1967): *Statistical Methods for Agricultural Workers*. 2nd Ed. ICAR, New Delhi, India.

Setia, R. K., Sharma, K. N. (2004): Vertical distribution of chemical pools of potassium and their relationship with potassium nutrition of wheat. *J. Indian Soc. Soil Sci.*, **52**, 469–472.

Sharma, B. D., Singh, R. (1982): Distribution of nitrate N and N uptake by rainfed maize as affected by method of application of urea. *J. Indian Soc. Soil Sci.*, **30**, 44–47.

Sharma, K. N., Bhandari, A. L., Kapur, M. L., Rana, D. S. (1985): Influence of growing various crops in five fixed cropping sequences on the changes in nitrate and total N content of soil. *J. Agri. Sci., Cambridge,* **109**, 281–284.

Singh, B., Sadana, U. S., Arora, B. R. (1991): Nitrate pollution of ground water with increasing use of N fertilizers in Punjab. *Indian Journal of Environment Health*, **33**, 516–518.

Singh, B., Singh, Y., Sekhon, G. S. (1995a): Fertilizer use efficiency and nitrate pollution of ground water in developing countries. *J. Contaminant Hydrology*, **20**, 167–184.

Singh, H., Sharma, K. N., Arora, B. S. (1995b): Influence of continuous fertilization to a maize-wheat system on the changes in soil fertility. *Fertilizer Res.*, **40**, 7–19.

Subbiah, B. V., Asija, G. L. (1956): A rapid procedure for the estimation of available N in soils. *Current Science,* **25**, 259–260.

Walia, O. S., Ahmed, N., Uppal, K. S., Rao, Y. S. (1998): Profile distribution of various forms of N and C/N ratio in some land forms of Bundelkhand region of Uttar Pradesh. *J. Indian Soc. Soil Sci.*, **46**, 193–198.

Williams, C. H., Steinberg, A. (1959): Soil sulphur fraction as chemical indices of available S in some Australian soils. *Australian J. Agri. Res.*, **10**, 349–351.

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EFFECT OF INTEGRATED USE OF *AZOTOBACTER* AND NITROGEN FERTILIZER ON YIELD AND QUALITY OF ONION (*Allium cepa* L.)

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A field experiment was conducted during the summer season of 2000–2001 at IARI, New Delhi using the onion cultivar Pusa Madhvi to identify a suitable *Azotobacter* strain and nitrogen level combination(s) for better yield and quality of onion. The treatments consisted of factorial combinations of four levels of nitrogen (0, 25, 50 and 75% recommended doses) and three *Azotobacter* strains (CBD-15, AS-4 and M-4) with two uninoculated controls, one with the full dose of N and the other without NPK. The results revealed that the application of 75% recommended N dose along with inoculation with CBD-15 or M-4 significantly increased the marketable yield and the nitrogen content in both leaves and bulbs, over the control with the full dose of nitrogen, whereas only 75% recommended N + CBD-15 led to a significantly increased total yield. Total soluble solids (TSS) and neck thickness were significantly reduced by the application of 50% recommended N dose along with inoculation with CBD-15 or M-4 compared with the uninoculated control with the full N dose. Inoculation with any of the *Azotobacter* strains along with 50 or 75% recommended N dose significantly reduced the sprouting loss during storage, while inoculation significantly reduced rotting and total losses when combined with 50 or 25% recommended N doses.

***Key words*:** *Azotobacter* strains, nitrogen, onion, yield, quality

Introduction

Onion (*Allium cepa* L.) is one of the most important vegetable crops grown and used throughout the world. As onion has a high nitrogen requirement, its productivity depends to a great extent on the use of optimum fertilizer rates.

Modern Indian agriculture depends heavily on the use of chemical fertilizers. However, in the present scenario the adverse effects of the indiscriminate use of chemical fertilizers on long-term soil health and environment have become immense and of late have received global attention. Moreover, the addition of inorganic fertilizer in higher doses constitutes one of the most expensive inputs, tending to make agriculture beyond the capacity of resource-poor farmers. An integrated approach involving nitrogen-fixing biofertilizers such as *Azotobacter* and chemical nitrogenous fertilizers can substantially minimize these problems besides boosting the yield and quality of the crop. However, there is a wide variation in the capacity of various strains of *Azotobacter* to exert their beneficial effect (Vinay et al., 1998). This study was therefore conducted to identify suitable *Azotobacter* strains and nitrogen level combination(s) for better yield and quality of onion.

Materials and methods

The experiment was conducted in the field using onion cultivar Pusa Madhvi during the summer season of 2000–2001 at IARI, New Delhi, on sandy loam soil having a pH of 7.9 and EC of 0.26. Four levels of nitrogen (0, 25, 50 and 75% recommended doses) and three *Azotobacter* strains (CBD-15, AS-4 and M-4) were combined factorially with two additional uninoculated controls, one with the full dose of N (standard practice) and the other without NPK (absolute control). A total of 14 treatments including controls were laid out in a randomized block design (RBD). The plot size was 4.68 m^2 with a spacing of 10 cm × 15 cm. Carrier-based (charcoal: soil, 3:1) inocula of each of the *Azotobacter* strains @ 500 g ha^{-1} were suspended in water to prepare a slurry. Seedlings were uprooted from the nursery beds and dipped in the respective *Azotobacter* strain slurry before transplanting in the main field. Only half the dose of nitrogen as urea was applied at the time of transplanting and the other half was applied in two equal splits at 30 and 50 days after transplanting. The full doses of phosphorus as single superphosphate and potassium as muriate of potash were applied to all the treatments at transplanting @ 50 kg P_2O_5 and 75 kg K_2O ha^{-1}, respectively. Besides total and marketable yields, data were recorded on quality parameters, namely neck thickness, total soluble solids (TSS), nitrogen content of different plant parts and shelf life of onion bulbs.

Results and discussion

Yields

The integration of chemical nitrogenous fertilizer with *Azotobacter* strains markedly influenced both total and marketable yields (Table 1). Supplementation of CBD-15 with 75% of the recommended dose of N increased the total yield of onion by 12.9% over the application of the full dose of nitrogen alone, whereas inoculation with M-4 or AS-4 along with the application of 75% of the recommended N dose and CBD-15 or M-4 along with application of 50% of the recommended N dose resulted in total yields which were at par with that of the standard practice, ascertaining that up to 50% of the recommended dose of nitrogenous fertilizer could be saved without significantly reducing the total yield obtained by applying the full dose of N alone. Similarly, Konde et al. (1978) and Ukey (1998) reported increased total yields in onion due to *Azotobacter* inoculation.

Treatment with 75% recommended N + CBD-15 or 75% recommended N + M-4 appreciably increased the marketable yield of onion bulbs over the control with the full N dose. These two treatments increased the marketable yield by 15 and 11.9%, respectively, over the standard practice. Inoculation with AS-4 along with 75% N dose and CBD-15 or M-4 along with 50% N dose resulted in marketable yields which were at par with that of the standard practice (Table 1). Similarly, Bhonde et al. (1997) reported the highest marketable yield (230.62 q ha^{-1}) of onions due to the combined application of *Azotobacter* and 50% of the recommended dose of nitrogenous fertilizer. The increase in yields was mainly attributed to the multiple effects of *Azotobacter,* which acts not only by fixing atmospheric nitrogen but also by suppressing pathogenic microorganisms, and by producing growth-promoting substances. Moreover, its role in the mineralization of phosphate and in the general improvement in plant nutrient uptake due to root proliferation might also have considerably contributed to the enhanced yields in the inoculated treatments in these experiments.

Nitrogen content in leaves and bulbs

The nitrogen content in bulbs was significantly increased due to supplementation of 75% the recommended N dose with either CBD-15 or M-4 over the control with the full dose of nitrogen (Table 1). These two treatments gave 0.24 and 0.16% higher nitrogen content in bulbs over the standard practice and 1.17 and 1.0% over the absolute control, respectively. A significant increase in the percentage nitrogen in the grain and stover of maize was also reported due to *Azotobacter* inoculation along with a moderate amount of nitrogenous fertilizer by Meshram and Shende (1982).

Table 1

Shoot and bulb N content and yields (q ha^{-1}) of onion as affected by combined application of *Azotobacter* strains and reduced nitrogen doses

Treatment	Nitrogen content (%)			Total yields	Marketable yields
	Leaves		Bulbs		
	45 DAT	90 DAT			
T_1	2.19	2.51	2.53	271.4	252.1
T_2	2.83	2.89	3.57	405.3	392.5
T_3	2.14	2.45	2.50	246.4	226.5
T_4	2.70	2.78	3.35	363.2	349.7
T_5	2.24	2.54	2.60	273.5	260.1
T_6	2.77	2.87	3.49	394.6	381.7
T_7	2.63	2.76	3.22	358.5	358.2
T_8	2.55	2.72	3.16	339.1	326.9
T_9	2.61	2.76	3.21	355.3	344.0
T_{10}	2.44	2.58	2.89	310.5	299.9
T_{11}	2.38	2.53	2.79	287.0	272.8
T_{12}	2.49	2.62	2.91	313.5	299.1
T_{13}	2.67	2.78	3.33	359.0	341.2
T_{14}	1.95	2.31	2.40	216.5	190.2
SEm±	0.02	0.02	0.04	13.89	13.41
C.D. (5%)	0.07	0.07	0.12	40.38	38.99

$T_1 = N_0$ + CBD-15; T_2 = 75% N + CBD-15; $T_3 = N_0$ + AS-4; T_4 = 75% N + AS-4; $T_5 = N_0$ + M-4; T_6 = 75% N + M-4; T_7 = 50% N + CBD-15; T_8 = 50% N + AS-4; T_9 = 50% N + M-4; T_{10} = 25% N + CBD-15; T_{11} = 25% N + AS-4; T_{12} = 25% N + M-4; T_{13} = Full dose of N without *Azotobacter*; T_{14} = Without NPK or *Azotobacter*

Similar to the nitrogen content in the bulbs, the nitrogen in the leaves at 45 days after transplanting (DAT) was markedly increased over both the controls and over other treatments due to the application of 75% of the recommended N dose supplemented with *Azotobacter* strains CBD-15 or M-4. These two treatments showed 0.16 and 0.10% higher nitrogen content in the leaves at 45 DAT over the standard practice and 0.88 and 0.82% over the absolute control, respectively. These treatments also resulted in significantly higher percentage nitrogen content in the leaves at 90 DAT over both the controls and all other treatments. Treatment with 75% recommended N + CBD-15 led to 0.11 and 0.58% higher nitrogen in the leaves at 90 DAT over standard practice and the absolute control, respectively, whereas 75% recommended N + M-4 gave 0.09 and 0.56% higher nitrogen in the leaves at 90 DAT over standard practice and the absolute control, respectively. Increased shoot nitrogen content was earlier reported in tomato by El-Shanshoury et al. (1989) due to inoculation with *Azotobacter chroococcum*. Similarly, Ahmad (1998) reported the highest leaf nitrogen content in mango after the application of 145 g N tree^{-1} + *Azotobacter* (CBD-15). The increase in nitrogen content in both the plant parts might be due to the better root development achieved as a result of inoculation with efficient strains, which led to enhanced nutrient uptake.

Storage quality

The supplementation of both the 25 and 50% recommended N doses with either CBD-15 or M-4 resulted in a marked reduction in the total loss after 3 months of storage over the control with the full dose of nitrogen (Table 2). The application of 25% of the recommended N dose along with inoculation with M-4 or CBD-15, and 50% of the recommended N dose along with M-4 reduced the total loss by 3.83, 3.53 and 3.50%, respectively, compared with the control with the full dose of nitrogen. The same treatments resulted in 4.86, 4.56 and 4.53% less total loss, respectively, as compared with the absolute control. However, after 5 months of storage, supplementation of 50 and 25% recommended doses of N with any one of the *Azotobacter* strains tested led to a great reduction in the total storage loss as compared to the total loss recorded for both controls. Thus, up to 75% of the recommended N dose could be saved together with better shelf life of onion through inoculation with *Azotobacter*.

Rotting losses were substantially reduced due to the supplementation of 50% recommended N dose with any of the *Azotobacter* strains after both 3 and 5 months of storage (Table 2). The reduction in rotting losses due to *Azotobacter* might be attributed mainly to the ability of the bacterium to produce antifungal and antibacterial substances which might have inhibited the growth of pathogenic organisms.

A significant reduction in sprouting losses after 3 months of storage was obtained due to the application of 50% of the recommended N dose along with inoculation with any of the strains and 75% recommended N dose with either

CBD-15 or M-4 as compared to both the controls. Likewise, after 5 months of storage, the supplementation of 50 and 75% recommended N doses with any of the strains appreciably reduced sprouting losses over standard practice as well as over the absolute control. Although literature specific to *Azotobacter* is not available, Ranpise et al. (2001) reported the significant effects of biofertilizers in reducing various storage losses and the present findings confirm their report. However, no marked differences between the treatments were observed with respect to physiological weight loss after 3 and 5 months of storage due to the combination of *Azotobacter* strains and nitrogen levels. Furthermore, no role of *Azotobacter* with respect to physiological processes in the bulb has so far been suggested that would support the present findings.

Table 2

Influence of combined application of *Azotobacter* strains and reduced nitrogen doses on shelf life of onion bulbs after 3 and 5 months of storage

	Storage losses (%)							
Treatment	3 months				5 months			
	Total loss	PLW	RL	Sprouting	Total loss	PLW	RL	Sprouting
T_1	15.30	3.40	8.73	3.27	42.00	17.07	16.30	8.63
T_2	13.83	2.87	8.42	2.37	38.23	17.32	15.26	5.18
T_3	15.47	2.88	9.30	3.28	43.57	18.24	16.77	8.89
T_4	14.97	3.53	8.87	2.57	39.40	18.13	15.57	6.33
T_5	15.23	3.40	8.57	3.17	40.80	15.96	16.05	8.54
T_6	14.31	3.37	8.50	2.43	37.90	16.90	15.30	5.70
T_7	12.17	5.13	5.43	1.60	34.10	20.28	9.63	4.18
T_8	13.00	4.13	6.57	2.30	36.17	18.70	12.40	5.07
T_9	11.80	3.83	6.10	1.87	31.63	16.54	10.70	4.36
T_{10}	11.77	1.60	7.33	2.83	34.43	14.03	12.83	7.57
T_{11}	12.67	2.10	7.60	2.97	35.00	13.75	14.23	7.82
T_{12}	11.47	1.80	6.90	2.77	33.33	13.43	12.69	7.27
T_{13}	15.30	3.87	8.30	3.13	40.50	16.39	15.13	7.98
T_{14}	16.33	2.70	9.70	3.93	44.47	17.58	17.37	9.52
SEm±	1.02	1.07	0.51	0.23	1.03	1.52	0.84	0.44
C.D. (5%)	2.97	3.10	1.47	0.66	3.00	4.43	2.44	1.27

For treatments see Table 1; *PLW and RL denote physiological loss in weight and rotting loss, respectively

Neck thickness and total soluble solids (TSS)

Narrow neck thickness is a desirable character in onion since it protects the bulbs against invading pathogens. On supplementing 50% of the recommended N dose with M-4 or CBD-15 and 75% of the recommended N dose with M-4, a significant reduction in onion neck thickness was obtained compared with the application of the full dose of nitrogen alone (Fig. 1). There was a marked increase in the total soluble solids (TSS) of onion bulbs due to inoculation with CBD-15 along with the application of 50% of the recommended

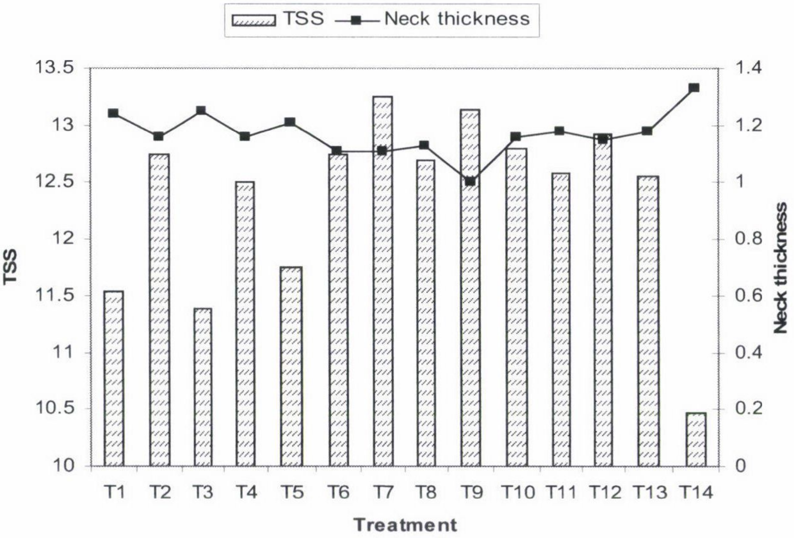

Fig. 1. Total soluble solids (%) and neck thickness (cm) of onion as influenced by combined application of *Azotobacter* strains and reduced nitrogen doses

N dose or M-4 along with 50 or 25% of the recommended N doses, compared with standard practice (Fig. 1). The application of the 50% nitrogen dose along with CBD-15 inoculation increased total soluble solids (TSS) by 5.25 and 26.36% over standard practice and the absolute control, respectively. Similarly, inoculation with M-4 along with 50 and 25% nitrogen doses increased total soluble solids (TSS) by 4.22 and 2.63%, respectively, over standard practice and by 25.12 and 23.21%, respectively, over the absolute control. Inoculation was more effective in increasing total soluble solids (TSS) with 50 and 25% nitrogen doses than with the 75% dose, which is also in agreement with the findings of Ahmad (1998), who ascertained maximum total soluble solids (TSS) in mango fruit due to inoculation with *Azotobacter* CBD-15 along with the application of 96 g N tree^{-1} rather than 145 g N tree^{-1}. Although inoculation with CBD-15 or M-4 gave better total soluble solids (TSS), inoculation with AS-4 at all the nitrogen levels also gave total soluble solids (TSS) values that were at par with that of standard practice. Similarly, Kumaraswamy and Madalageri (1990) reported that tomato plants treated with *Azotobacter* produced fruit with high total soluble solids (TSS). Ukey (1998) also reported an average 2.56% increase in total soluble solids (TSS) in onion with *Azotobacter* + 50% reduced nitrogen level over its uninoculated counterpart (50% reduced nitrogen alone).

References

Ahmad, F. M. D. (1998): *Response of* Azotobacter chroococcum *in integrated nutrient management in mango cv. Amarpali.* Ph.D. Thesis. I.A.R.I, New Delhi.

Bhonde, S. R., Sharma, S. B., Chougule, A. B. (1997): Effect of biofertilizer in combination with nitrogen through organic and inorganic sources on yield and quality of onion. *National Hort. Res. and Development Foundation,* Nasik, Maharashtra, **17**(2), 1–3.

El-Shanshoury, A. R., Hassan, M. A., Abdel-Ghaffar, B. A. (1989): Synergistic effect of vascular-arbuscular-mycorrhizas and *Azotobacter chroococcum* on growth and nutrient contents of tomato plants. *Phyton-Horn.,* **29**, 203–212.

Konde, B. K., Desai, J. N., More, B. B., Shende, P. A. (1978): Effect of *Azotobacter* on growth and yield of onion under field condition. *Food Farming and Agriculture,* **9**(7), 185–187.

Kumaraswamy, D., Madalageri, B. B. (1990): Effect of *Azotobacter* inoculation on tomato. *South Indian Hort.*, **38**, 345–346.

Meshram, S. U., Shende, S. T. (1982): Total nitrogen uptake by maize with *Azotobacter* inoculation. *Plant and Soil*, **69**, 275–279.

Ranpise, S. A., Birade, R. M., Patil, B. T., Sawant, S. V. (2001): Factors affecting the storage of onion: A review. *The Orissa Journal of Horticulture*, **29,** 1–12.

Ukey, R. N. (1998): *A pragmatic approach for supplementation of chemical fertilizers with biofertilizers to onion crops* (*Allium cepa* L.). Ph.D. Thesis. I.A.R.I., New Delhi.

Vinay, G., Gupta, R. D., Bharadwaj, K. K. R. (1998): Abundance of *Azotobacter* in great soil groups of North West Himalayas. *J. of Indian Soc. Soil Sci.*, **46,** 379–383.

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IMPACT OF COMPOSTS PRODUCED FROM WASTE OF ANIMAL ORIGIN ON THE BIOLOGICAL ACTIVITY OF SOILS

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Due to changes in the Hungarian legislation, the ATEVSZOLG Corporation, which treats waste of animal origin, has started to search for a new way to dispose and reuse this waste by recycling it without the loss of materials produced at high cost from the natural cycle. Since this waste contains a high concentration of fat, one major objective of the composting experiment was to investigate the effect of composts with high fat contents on the biological activity of the soil. The other aim was to investigate the impact of sterilising heat treatment and of high temperature conditions during the composting process on the number of pathogenic microbes, which are common in waste of animal origin. The quality and quantity of the fat in the soil samples were measured using a gas chromatograph. The effect of the high fat content on the biological activity of the soil was measured as the difference between the control and the treated soil samples for CFU number of fat-degrading microbes and the difference in the biological activity of the samples in an Oxi-Top soil respirator system.

The effect of heat treatment on pathogenic microbes was investigated on the basis of the number of *Clostridium*, faecal coliforms and *Pseudomonas aeruginosa* microbes. The results showed that the high fat content deposited with the composts was well utilised, and that its degradation did not cause a problem for the microbes living in the soil. This was proved both by the results of the CFU experiments and by the parameters in the Oxi-Top soil respirator system. The heat treatment successfully decreased the number of pathogenic microbes to a low risk level. The results indicated that the mixing of the heat-treated, sterilised basic materials of the composts with untreated, non-sterilised materials such as sewage sludge should be avoided, due to the risk of re-infecting the compost with pathogens. The composts produced from animal waste using the heat treatment developed by the ATEVSZOLG Corp. have the same infection risk as the composts produced from animal manure or sewage sludge.

Key words: waste of animal origin, composting, fat content, pathogenic microbes, biological activity

Introduction

The 300,000 tonnes of waste of animal origin (excluding animal and liquid manure) produced each year in Hungary is divided into three categories. The treatment, recycling and disposal of the waste are dependent on the categories. The second category includes high-risk materials such as manure, liquid manure, rumen, rumen waste, slaughterhouse sewage sludge and protein fodder of animal origin. The decree authorizes two methods, composting and biogas production, for the recycling of the waste in Category 2.

Considering the decline in the livestock population in Hungary since 1989, it might be expected that the amount of animal waste should have decreased. However, the decrease in the livestock population has slowed down and the Hungarian meat industry imports animals to replace the shortage. The waste arising during the slaughtering of imported animals has to be disposed of in Hungary. Any reduction in the waste quantity has been compensated for by the introduction of strict veterinary legislation, the HACCP system and stricter rules for the treatment of sewage.

Animal waste is a potentially infectious material, which must be treated using special methods, which are very expensive. Research on how the risk of infection can be reduced and on how this waste can be recycled into the natural cycle is thus given priority.

The legislation allows animal waste to be disposed of by composting, burning or by using it to produce biogas.

The role of composting and manuring has changed, the main role no longer being the supply of nutrients, but the recycling of organic matter into the natural cycle, while the amelioration of the soil structure is also gaining importance.

Factors influencing the composting process

During composting many factors must be considered to achieve optimal conditions. One of the most important is the nature of the basic material. Animal waste contains water, protein, fat and ash materials.

The water content is easily controlled, while ash materials have little influence on the composting process. The high protein content is not a problem, because proteins are easily degraded by microbes during composting, and proteins have an optimal C/N ratio (Hegedűs et al., 1998).

The degradation of the high fat content is more problematic and is also more difficult than that of the other materials. The microbes responsible for the composting process degrade fats, which have long carbon chains, either after or simultaneously with the proteins. Any materials present in a high concentration may be prohibitive for microbial processes.

Reduction in the number of pathogenic microorganisms

The animal waste deposited on animal waste dumps, recycled as biogas or compost and reused as soil ameliorants or manure must be sampled immediately after heat treatment and must be free of heat-resistant pathogenic bacterium spores.

According to legislation on the storage, trade and use of yield-enhancing materials, a microbiological analysis must be carried out on all composts before they can be marketed. During this analysis the number of faecal coliforms, faecal *Streptoccoccus*, *Salmonella* sp. and human intestinal parasite eggs is determined. The legal limit for faecal coliforms is less than 10 cells/g.

During the composting process the aim is not only to recycle organic material, but also to produce compost with no risk for public health. The temperature conditions occurring during composting have a very important sterilising effect. There is a very high risk of environmental infection by the residual organic waste from agriculture and the food industry. The high temperature occurring during the degradation phase of the composting process guarantees the destruction of pathogenic microbes. This temperature must reach 55°C for a certain period if the process of disinfection is to be completed (Maier et al., 1999). The pathogen organisms, parasites and weed seeds are not entirely destroyed during the composting process, so the compost is not sterile, but it has a decreased infection risk.

During the composting of sewage sludge it was observed that after the lethal high temperature period, certain pathogenic microbes reinfected the compost heap (Maier et al., 1999). This phenomenon could be caused by the following:

1. The temperature did not reach the critical 55°C in the compost heap, or the critical temperatures was not maintained for a long enough period. It was stated by Maier et al. (1999) that temperatures above 53°C for 3 days were sufficient to destroy pathogenic organisms.

2. Because of the inadequate technology or failure to homogenise the compost heap, there are residual focal points where pathogenic organisms can survive the high temperature period. The decreased natural microbial population as a consequence of the high temperature period means that there is no limit to the multiplication of the pathogens. The incomplete composting process creates optimal conditions and free living space for the pathogen microbes, which can easily multiply in the compost (Shuval et al., 1991).

3. Reinfection with pathogens occurs due to transmission by birds, rodents, insects or humans, not from the compost itself.

The pathogenic microbes occurring in both composts and animal waste can be classified on the basis of two qualities. Some bacteria are capable of producing asexual cells, or spores, which are capable of surviving under various environmental conditions. If such a spore is produced inside the cell, it is called an endospore. Endospore production takes place when the resources for active growth are exhausted. Endospores are extremely resistant to heat, drying, radioactivity and various chemicals. This resistance is due to the cortex surrounding the spore, which is made up of calcium dipylcolynate, and to the dehydrated state of the spore. The spores are capable of surviving for hundreds or thousands of years and if conditions become optimal the spores are activated and start to multiply. Microbes producing spores are dangerous because they are able to survive extreme environmental conditions, such as high temperatures. Some members of the *Clostridium* and *Bacillus* genera can survive temperatures of 70–80°C for a period of up to 15 minutes. Spores of *Bacillus subtilis* were reported to survive temperatures of 100°C (Minnich, 1979). Microbes unable to produce spores act as common cells when affected by heat. The heat inactivates the enzymes, and when the effect is intense enough, the enzymes are totally destroyed and the cell (bacterium) dies. Pathogenic microbes can also be classified into two groups on the basis of the type of pathogenesis. Obligate pathogens can cause illness in healthy people just as well as in people with a weak immune system, while facultative pathogens can only cause illness in people with a weak immune system (due to burns, injuries, operations, antibiotic treatment, etc.). These include members of the *Acinetobacter*, *Aeromonas*, *Pseudomonas* genera, etc.

In the United States of America the main cause of food poisoning is *Clostridium perfringens* (Madigan et al., 1998). Hungarian legislation requires all waste of animal origin to be sterilized by heat treatment to ensure the complete elimination of this pathogen.

Faecal coliform bacteria are facultative pathogens common in inadequately treated composts and sewage sludge. The exotoxin produced by one *E. coli* strain causes 20,000 cases of food poisoning yearly in the USA (Madigan et al., 1998). Faecal coliforms are indicators of other pathogen microbes such as the *Salmonella* genus. According to the results of experiments on the depositing of sewage sludge on agricultural lands, the presence of faecal coliforms is correlated with the presence of *Salmonella* strains (Gibbs et al., 1997). The Environment Protection Agency of the USA (EPA) requires tests for *Salmonella* strains when the number of faecal coliforms increases to 1000 CFU/g in the case of composts produced from sewage sludge. It was found in several experiments that *Salmonella* strains were absent from the compost sample when the faecal coliform number was less than 1000 CFU/g, but were present when the faecal coliform number exceeded this figure (Roger, 1993).

The *Pseudomonas aeruginosa* bacterium is a ubiquitous facultative pathogen strain, of increasing interest to scientists. It is widespread in the human environment. It can be found in domestic sewage, surface water bodies and spas. It is able to multiply at a high temperature (35–42°C); it is also common in thermal waters, deep boreholes, cooling waters and distilleries. The infective cell number of *Ps. aeruginosa* is low, having a threshold value of 10^2 (Némedi et al., 1998). The strain is able to resist antibiotics and germicides due to R-factor plasmid transport (Madigan, 2000) and it is very resistant to extreme environmental conditions.

Materials and methods

The composted materials were slaughterhouse waste, slaughterhouse sludge and meat meal. The first step was to sterilise the basic materials of the composts, as required by Hungarian legislation, using the physico-chemical sterilising method developed and patented by the ATEVSZOLG Corp. The waste was ground to a size of 50 mm and heated to 133°C at a pressure of 3 bar for 20 minutes in a disinfector for sterilisation, after which it was no longer classified as hazardous.

In the following step the relative water content of the sterilised material is reduced by mixing with a material favourable for composting, such as straw. The basic material thus produced is not classified as hazardous or infectious. Some of the materials were mixed with unsterilised slaughterhouse sludge, so that the sludge was only subjected to the temperature conditions occurring during the composting process. This meant that the infection risk increased to a considerable extent. The content of the composts and the production method can be found in Table 1. Composting takes place in compost piles covered with polyethylene and equipped with an efficient aeration and moisture control system, assuring rapid, optimum composting.

After the composting process the maturation period differed for the individual piles. The composts were deposited at 50 t/ha and 200 t/ha rates on 20 plots at the experimental area of the Research Institute for Soil Science and Agricultural Chemistry of the Hungarian Academy of Sciences in Őrbottyán on 15th October 2002. There were 4 control plots without compost. The plots measured 5 × 8 m, and were cultivated suitably for the season and for the crop. The soils of the plots were sampled after harvesting the crops, using the normal agricultural sampling method. Samples were taken from the ploughed layer (30 cm), omitting a 1 m border, at 15 points, then homogenised to obtain a mean sample, which was analysed in the laboratory of the Department of Environmental Protection in Gödöllő.

The *Clostridium perfringens* and faecal coliform number of the samples was determined in the Microbiology Laboratory of the National Public Health and Medical Officer Service. The qualitative and quantitative fat content of the samples was analysed by gas chromatography in the Analytical Laboratory of Corvinus University, Budapest. The CFU, *Pseudomonas aeruginosa* and fat-degrading microbe number and the biological activity of the treated soils was determined by the Department of Environmental Protection.

The biological activity was measured in an Oxi-Top soil respirometer. During soil respiration the microbes in the medium consume oxygen and produce CO_2. The product (CO_2) is adsorbed in NaOH adsorbent, and this process causes a vacuum in the vessel, which is measured by the Top-C measuring head. The pressure change is in linear correlation with the oxygen consumption (biological activity) of the sample. The data can be retrieved and processed with an OC 100 controller in the form of tables and diagrams.

Table 1

Composts used in the experiment

Experiment code	Basic material of the compost	Composting period	Quality of the compost when distributed
4.8	Slaughterhouse waste compost + sewage sludge	Aeration: 2 months; Maturation: 10 months	Odourless, well matured, small fragments, good homogenisation
4.9	Meat meal + straw (1:0.5)	Aeration: 6 weeks; No maturation	Malodorous; fragments not homogeneous; 10–20 cm meat meal sods
4.10	Meat meal + straw (1:0.5)	Aeration: 6 weeks; Maturation: 6 months	Malodorous; fragments not homogeneous; 10–20 cm sods
4.11	Slaughterhouse waste + straw	Aeration: 2 months; Maturation: 8 months	Malodorous; fragments not homogeneous, 10–20 cm sods
4.12	Meat meal	No composting occurred	Meat meal

Results

Effect of composts with high fat content on soil and soil life

Gas chromatography measurements indicated that the fat content of treated soils was similar to that of the untreated control; in fact it was higher in one of the untreated soil samples than in the treated soils (Table 2). In composts produced from heat-treated animal waste with a high fat content (20%) the fat content was degraded and transformed during the composting process by microorganisms.

The total living cell number was an order of magnitude higher in treated soils than in untreated control soils (Table 3). This could be attributed to the increased organic matter content of the soil, making it more favourable for microbes. This increase in the total number of living cells is a sign of the intensification of biological activity in the treated soils. The treatment also caused an increase in the number of fat-degrading microbes compared to the control soils. There were only five cases where the number of fat-degrading microbes in the treated soil samples was greater than in the control soils (Table 3), suggesting that during the processing of the compost a balance is established among the microbe populations in the soil, where fat-degrading microbes play an important but not exclusive role. Changes in the total living cell number and the total number of fat-degrading microbes indicate that treating soils with composts with a high fat content has a stimulating effect on the multiplication and metabolism of soil-borne microbes.

This is confirmed by the results of soil biological activity analysis. In the Oxi-Top soil respirometer system the biological activity of the treated soils exceeded that of the control soils by 50–100% (Table 3). This increase amounted to 20–30% for soils treated with 50 t/ha compost, while the biological activity was 2–3 times higher in soils treated with 200 t/ha compost, compared with the untreated control soils (Figs. 1 and 2).

Table 2

Fat content (%) of the composts and treated soils, determined by gas chromatography

Experiment code	DMC	FCC	FCS		AFC
			50 t/ha	200 t/ha	
4.8	38.9	1.36	0.04	0.08	0.06
4.9	45.8	7.39	0.08	0.035	0.06
4.10	60	0.47	0.085	0.06	0.06
4.11	55.8	1.03	0.095	0.11	0.06
4.12	95	12.80	0.075	0.10	0.06

DMC: Dry matter content of the composts %; FCC: Fat content of the composts as a % of dry matter; FCS: Fat content of treated soil samples determined by gas chromatograph as a % of dry matter; AFC: Average fat content of untreated control soil samples as a % of dry matter

Table 3
Results of analysis on the effect of high fat content

Compost	Treatment	Sample code	TNM	TNFM	FCS	OTBA
No compost	Control	4.8 1/I	2.67×10^6	1.97×10^6	0.06	230.5
		4.8 1/V	6.56×10^6	1.26×10^6	0.06	261.3
	Control	4.9 1/II	8.82×10^6	9.00×10^6	0.06	112.7
		4.9 1/IV	9.40×10^6	8.23×10^6	0.06	194.7
Slaughterhouse waste compost + sewage sludge	50 t/ha	4.8 3/III	8.83×10^6	1.68×10^6	0.04	420.1
		4.8 3/IV	1.07×10^7	5.62×10^6	0.04	368.9
	200 t/ha	4.8 5/I	9.48×10^6	1.80×10^6	0.08	389.4
		4.8 5/III	2.23×10^7	4.58×10^6	0.08	179.3
Meat meal + straw without maturation	50 t/ha	4.9 3/II	1.97×10^7	2.83×10^6	0.08	230.5
		4.9 3/III	1.57×10^7	1.52×10^6	0.08	189.6
	200 t/ha	4.9 5/I	2.94×10^7	2.89×10^6	0.035	527.7
		4.9 5/III	3.39×10^7	5.60×10^6	0.035	507.2
Meat meal + straw with maturation	50 t/ha	4.10 3/III	7.13×10^7	5.9×10^6	0.085	548.2
		4.10 3/IV	1.92×10^7	9.25×10^6	0.085	496.9
	200 t/ha	4.10 5/I	2.386×10^7	7.06×10^6	0.06	563.5
		4.10 5/II	3.30×10^7	5.76×10^6	0.06	481.6
Slaughterhouse waste + straw	50 t/ha	4.11 3/II	4.85×10^7	9.78×10^6	0.095	174.2
		4.11 3/IV	1.47×10^7	2.36×10^6	0.095	450.8
	200 t/ha	4.11 5/I	1.0×10^8	2.88×10^6	0.11	768.5
		4.11 5/III	6.66×10^8	3.96×10^6	0.11	988.8
Meat meal	50 t/ha	4.12 3/II	3.52×10^7	1.65×10^6	0.075	123.0
		4.12 3/IV	1.75×10^6	3.78×10^6	0.075	133.0
	200 t/ha	4.12 5/I	8.50×10^6	6.1×10^6	0.10	338.1
		4.12 5/III	5.93×10^6	5.06×10^6	0.10	450.8

TNM: Total number of microorganisms (CFU/g); TNFM: Total number of fat-degrading microbes (CFU/g); FCS: Fat content of the soil sample as a % of dry matter determined by gas chromatography; OTBA: Oxi-Top Biological activity (hPa); CFU: Colony Forming Unit

Due to the composts the biological activity of the sandy experimental soil, which had low humus content, increased by 50–100% and the high fat content of the deposited composts was readily degraded and digested by the microbes.

Role of composts in the transmission of pathogenic microbes

The presence of *Clostridium perfringens* was only detected in treated soils, in 50% of which the *Clostridium perfringens* number exceeded the legally permitted limit. This was particularly noticeable in Experiment 4.8, where compost made of slaughterhouse waste was mixed with untreated sewage sludge after the compulsory heat treatment. In these samples the *Clostridium perfringens* number ranged from 6–19 CFU/g. A low number of *C. perfringens* was also recorded in other treated samples due to the fact that the composts were not well matured (Table 4).

The number of faecal coliforms did not exceed the legal limit (10 CFU/g soil) in any sample. This value only exceeded 1 CFU/g in two samples (4.11.5/III and 4.8.1/I). In 80% of the samples less than 0.18 CFU/g were detected (Table 4). The analyses revealed no difference in faecal coliform number between the treated and control soils. Due to the heat treatment and the temperature conditions occurring during the composting process, the composts were free of faecal coliforms and other non-spore-forming pathogenic microbes, so treating soils with composts produced from waste of animal origin involved no risk of transmitting faecal coliforms.

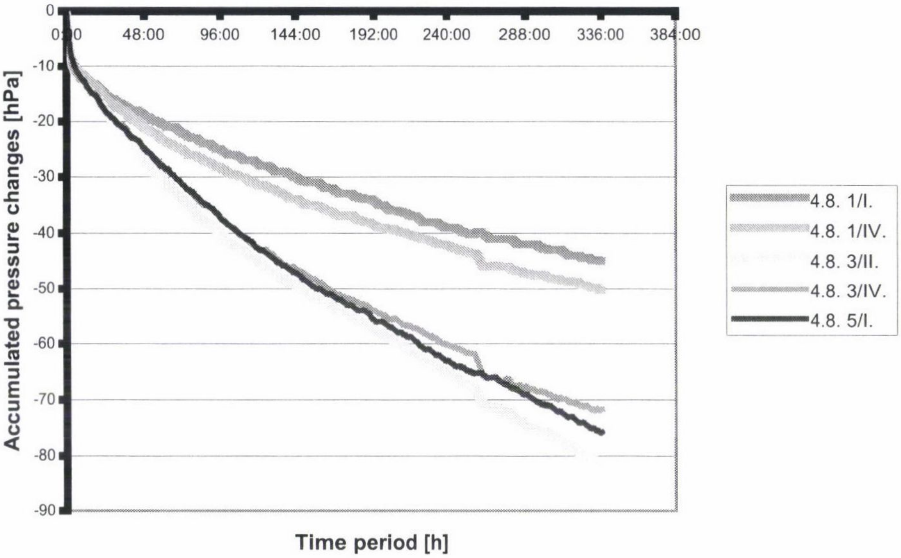

Fig. 1. Biological activity of soil samples from Experiment 4.8

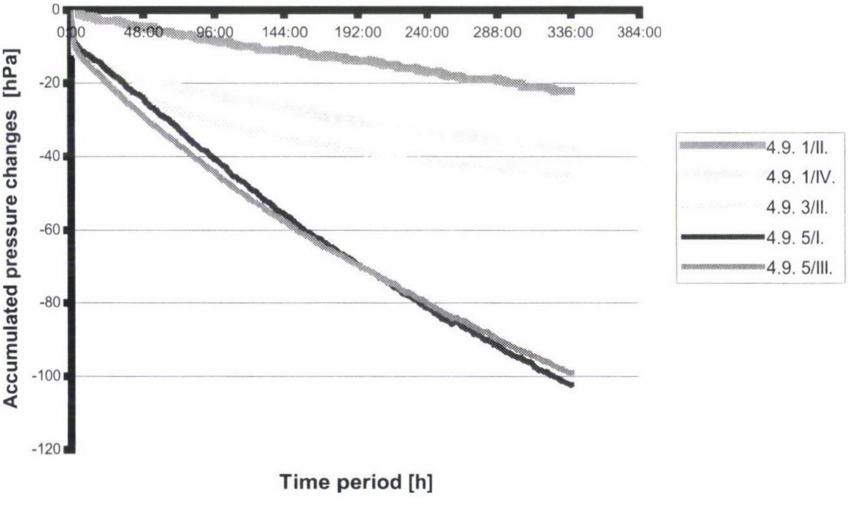

Fig. 2. Biological activity of soil samples from Experiment 4.9

Table 4

Pseudomonas aeruginosa, Clostridium perfringens and faecal coliform number in the soil samples

Compost	Treatment	Sample code	*Pa* No.	*Cp* No.	FCN
	Control	4.8 1/I	0	0	1.4/g
		4.8 1/IV	0	1	<0.18/g
	Control	4.9 1/II	0	0	<0.18/g
		4.9 1/IV	0	0	<0.18/g
Slaughterhouse waste compost + sewage sludge	50 t/ha	4.8 3/II	0	1	<0.18/g
		4.8 3/IV	0	7	<0.18/g
	200 t/ha	4.8 5/I	0	6	<0.18/g
		4.8 5/III	1000	19	<0.18/g
Meat meal + straw without maturation	50 t/ha	4.9 3/II	1	1	<0.18/g
		4.9 3/III	0	0	<0.18/g
	200 t/ha	4.9 5/I	1	1	<0.18/g
		4.9 5/III	10	1	<0.18/g
Meat meal + straw with maturation	50 t/ha	4.10 3/III	0	0	<0.18/g
		4.10 3/IV	0	0	0.2/g
	200 t/ha	4.10 5/I	0	0	0.2/g
		4.10 5/II	0	3	<0.18/g
Slaughterhouse waste + straw	50 t/ha	4.11 3/II	0	1	0.45/g
		4.11 3/IV	1	0	0.83/g
	200 t/ha	4.11 5/I	0	1	<0.18/g
		4.11 5/III	1	2	1.4/g
Meat meal	50 t/ha	4.12 3/II	0	0	0.2/g
		4.12 3/IV	0	0	0.45/g
	200 t/ha	4.12 5/I	0	0	<0.18/g
		4.12 5/III	0	0	<0.18/g

Pa No.: *Pseudomonas aeruginosa* number (CFU/g); *Cp* No.: *Clostridium perfringens* number (CFU/g), permitted limit: 0 CFU/g; FCN: Faecal coliform number (CFU/g), permitted limit: <10 CFU/g

The incidence of *Pseudomonas aeruginosa* exhibited no correlation with the compost application rate (Table 4). According to the results the *Ps. aeruginosa* strains isolated from the treated plots represented no health risk, because there was only one case (10^3 cell) where the infective cell number (10^2 cell) was exceeded, and this was a sample where non-sterilised sewage sludge was mixed with the sterile compost, thus increasing the risk of reinfection.

Discussion

Composts with a relatively high fat content (0.47–12.8 % dry matter) have no inhibitory effect on soil life, as confirmed by the degradation of the fat content (Table 4) and the increase in the biological activity (oxygen consumption) of the soil recorded in the Oxi-Top soil respirator system (Table 4).

The compulsory heat treatment of waste of animal origin and the temperature conditions during the composting process resulted in a decrease in the number of pathogenic microbes.

Mixing waste of animal origin, which has already been heat-treated, with high-risk non-sterilised waste such as sewage sludge, should be avoided. Composts produced from waste of animal origin contain a high concentration of easily degradable organic matter. This, together with the decreased number of microbes resulting from compulsory heat treatment and the temperature conditions during the composting process, may lead to the re-infecton of the compost with pathogenic microbes. The risk of infection from composts produced from waste of animal origin is no greater than that of composts produced from sewage sludge and animal manure. It is advisable to use this compost under crops not intended for direct human consumption (such as sunflower, sugar beet, etc.).

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References

Gibbs, R. A., Hu, C. J., Ho, G. E., Unkovich, I. (1997): Regrowth of faecal coliforms and *Salmonellae* in stored biosolids and soil amended with biosolids. *Water Science Technology*, **35**, 269–275.

Hegedűs, M., Schmidt, J., Rafai, P. (1998): Az állati eredetű melléktermékek kezelése. (*Treatment of Byproducts of Animal Origin.*) Mezőgazdasági Kiadó, Budapest, 256 p.

Madigan, M. T. (2000): Prokaryotic Diversity: Bacteria. pp. 453–544, 789–791, 986–988. In: Madigan, M. T., Martinko, J. M., Parker, J. (eds.), *Brock Biology of Microorganisms*. Ninth edition. Prentice-Hall International, London.

Maier, R. M., Pepper, I. L., Gerba, C. P. (1999): *Environmental Microbiology*. Academic Press, New York, pp. 348, 530.

Minnich, J. H. (1979): *The Rodale Guide to Composting*. Rodale Press, Emmaus, PA, USA, 315 p.

Némedi, L., Jánossy, L., Andrik, P., Kádár, M. (1998): Közegészségügyi környezetbakteriológia. (*Environmental bacteriology for public health.*) In: Andrik, P. et al. (1998): Környezetbakteriológia. 2. (bővített) kiadás, Környezetgazdálkodási Intézet, Budapest, pp. 149–247.

Roger, T. H. (1993): *The Practical Handbook of Compost Engineering*. Lewis Press, Torrance, CA, USA, pp. 162–200.

Shuval, H., Jodice, R., Consiglio, M., Spaggiarri, G., Spigoni, C. (1991): Control of enteric microorganisms by aerobic-thermophilic co-composting of wastewater sludge and agro-industry wastes. *Water Science Technology*, **24**, 401–405.

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Short communication

HERBICIDE TOLERANCE OF MARTONVÁSÁR MAIZE GENOTYPES

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A comparison was made of herbicide tolerance results for two years, one dry (2003) and one wet (2004). The maximum permitted dose and twice this rate of the herbicides (*mesotrione, mesotrione + atrazine, nicosulfuron, rimsulfuron*) were sprayed on inbred maize lines in the 7–8-leaf stage. The effect of the herbicides on 20 inbred lines was evaluated on the basis of visible phytotoxic symptoms. In the dry year the greatest damage, averaged over the inbred lines, was caused by the double rate of *rimsulfuron* and *nicosulfuron*, but the plants had overcome this by the end of the vegetation period. In 2004 the cool wet spring weather retarded the metabolic processes of maize, leading to greater phytotoxic damage. The most severe symptoms were observed for the double rate of *mesotrione + atrazine*. The phytotoxic damage caused by the "normal" rates applied in commercial maize production was overcome by the lines in the wet year, too. Despite the initial visible phytotoxic damage, none of the herbicides caused significant differences in grain yield between the control and the single or double rates of treatment.

Key words: maize inbred lines, herbicide tolerance, post-emergence.

Introduction

Since herbicide active agents were first discovered and widely introduced in the late 1940s, they have become an indispensable means of weed control. Different varieties of crops, including various genotypes of maize, have very different levels of tolerance to the authorised herbicides. The inbred lines used to produce maize seed are much more sensitive to external environmental effects than hybrids. Differences in the tolerance of various maize genotypes have been reported to herbicides of the 2,4-D (Miller, 1958), atrazine (Andersen, 1964; Eastin et al., 1964; Thomson et al., 1970) and sulfonylcarbamide type (Eberlein et al., 1989; Green and Ulrich, 1993, 1994; Green, 1998; Bónis et al., 2000, 2004; Bónis et al., 2003; Bunting et al., 2004).

Apart from the genotype, the phytotoxic effect of herbicides also depends to a great extent on external environmental factors (Berzsenyi et al., 1997; Burt and Akinsorotan, 1976). Under cold, wet conditions the post-emergence application of atrazine, which is superselective for maize, nevertheless caused intense damage to the crop (Thomson et al., 1970). Investigations on other herbicides confirmed the role of temperature (Penner, 1971) and soil moisture (Kern et al., 1975) in determining the extent of phytotoxic damage.

It was stated by Green (1998) that in the past herbicides were selected over a longer period of time, leading to the elimination of sensitive germ plasm from production, while nowadays the introduction of both herbicides and hybrids has speeded up, so this process of elimination is no longer ensured.

The aim of the present work was to compare the damaging effect of various post-emergence herbicides in a dry hot year and in a cool wet year, on the basis of visible phytotoxic symptoms.

Materials and methods

Investigations were made in 2003 and 2004 on the tolerance of 20 inbred maize lines grown on chernozem soil (Calciustoll) in Martonvásár to herbicides applied post-emergence. The experiment was set up in a two-factor split-plot design with two replications, with an untreated control plot for each treatment. The active agents in these herbicides were as follows: *mesotrione, mesotrione + atrazine, rimsulfuron, nicosulfuron.* The treatments are presented in Table 1. Of these herbicides, only *mesotrione* and *mesotrione + atrazine* are authorised for use in maize seed production. However, due to the frequent appearance and multiplication of many troublesome weeds on seed production fields, it is important to test herbicides that are at present only authorised for use on fields sown for grain and silage. The herbicides were applied post-emergence, in the 7–8-leaf stage of maize (BBCH 17-18), using the maximum dose permitted in the licence and double this dose. During the course of the vegetation period, visible phytotoxic damage was scored 9 (in 2003) and 14 (in 2004) days after the treatment using a 0–100 scale on which 0 indicated undamaged and 100 killed plants. The phytotoxicity percentage combines the number of killed plants and the extent of leaf damage due to scorching in a single parameter.

The weather in 2003 was hot and dry, with around 100 mm less rainfall than average during the vegetation period (–43%), the greatest deficiency being suffered at sowing and during the first three, crucial months of maize development. The quantity of rainfall in 2004 was similar to the many years' mean, but the mean temperature was 1.5–2.0°C lower than normal for the region.

Table 1
Treatments applied in the experiment

No.	Treatment	Dose, g ai ha^{-1}
1.	Mesotrione	144
2.	Mesotrione	288
3.	Mesotrione + atrazine	144 + 1000
4.	Mesotrione + atrazine	288 + 2000
5.	Nicosulfuron	40
6.	Nicosulfuron	80
7.	Rimsulfuron	15
8.	Rimsulfuron	30

Results and discussion

The percentage occurrence of visible phytotoxic symptoms, averaged over the inbred lines, is presented in Figure 1. In the hot, dry weather of 2003 the level of phytotoxicity nine days after treatment was classified as mild to moderate (5–10%) on the EWRC scale. For all the herbicides the double rate caused more intense symptoms on the inbred lines. The greatest damage was caused by the double rate of *rimsulfuron* and *nicosulfuron*, though these gradually disappeared during the course of the vegetation period.

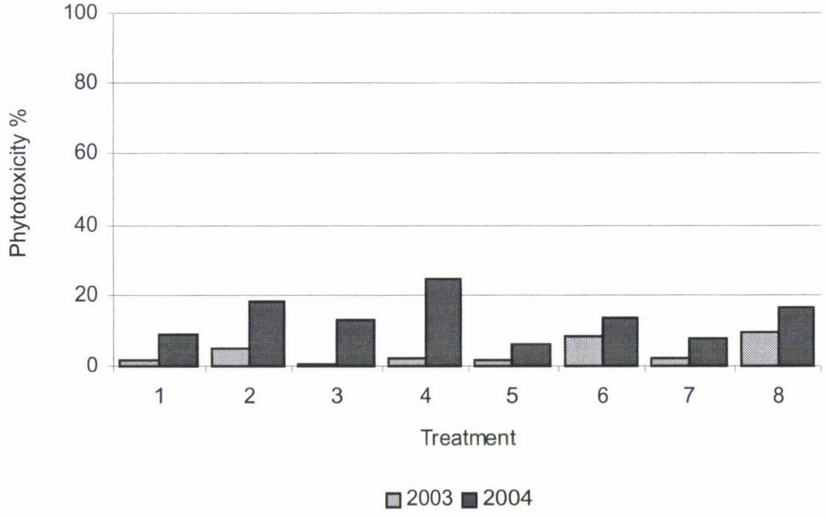

Fig. 1. Phytotoxic damage caused by herbicides on inbred maize lines. Martonvásár, 2003–2004

Due to the cool wet weather in spring 2004 the visible phytotoxic symptoms were more severe than in 2003. The greatest damage was caused by double rates of *mesotrione + atrazine,* and *mesotrione* or *rimsulfuron* alone. The cool weather retarded the detoxification process, leading to the accumulation of the active agents in maize. This could explain the fact that the degree of phytotoxic damage approached the moderate level on the EWRC scale in the case of the double rate.

The grain yield was not significantly reduced by any of the herbicide treatments.

Conclusions

It is a well-known fact that maize genotypes exhibit very different levels of tolerance to herbicides. In addition to the continuous testing of new inbred lines and newly introduced herbicides, attention must also be paid to the influence of external environmental conditions (weather) on the development and intensity of the phytotoxic damage caused by herbicides. The same genotype may exhibit much more severe visible damage in unfavourable years than under average climatic conditions that promote the natural detoxification of herbicides.

The results achieved over two years prove that, for certain herbicides, inbred lines are capable of tolerating the phytotoxic damage caused by herbicide treatment without loss of yield.

References

Andersen R. N. (1964): Differential response of corn inbreds to simazine and atrazine. *Weeds*, **12,** 60–61.

Berzsenyi Z., Győrffy, B., Árendás, T., Bónis, P., Lap, D. Q. (1997): Studies on the phytotoxicity of herbicides in maize (*Zea mays* L.) as affected by temperature and antidotes. *Acta Agron. Hung*., **45,** 443–448.

Bónis, P., Árendás, T., Berzsenyi, Z., Marton, L. C. (2004): Herbicide tolerance studies on maize inbred lines. *Z. PflKrankh. PflSchutz, Sonderh.*, **XIX**. 901–907.

Bónis, P., Árendás, T., Marton, L. C. (2003): Field tests on the herbicide tolerance of various maize genotypes. *J. of Agric. Sci. Debrecen.*, **11,** 21–23.

Bónis, P., Árendás, T., Berzsenyi, Z., Marton, L. C. (2000): Kukorica hibridek szülői komponenseinek herbicid toleranciája. (Herbicide tolerance of parental components of maize hybrids.) *Növényvédelem*, **12,** 633–638.

Bunting, J. A., Sprague, C. L., Riechers, D. E. (2004): Physiological basis for tolerance of corn hybrids to foramsulfuron. *Weed Sci.*, **52,** 711–717.

Burt, G. W., Akinsorotan, A. O. (1976): Factors affecting thiocarbamate injury to corn I. Temperature and soil moisture. *Weed Sci.*, **24,** 319–321.

Eastin, E. F., Palmer, R. D., Grogan, C. O. (1964): Mode of action of atrazine and simazine in susceptible and resistant lines of corn. *Weeds*, **12,** 49–52.

Eberlein, C. V., Rosow, K. M., Geadelmann, J. L., Openshaw, S. J. (1989): Differential tolerance of corn genotypes to DPX-M6316. *Weed Sci.*, **37,** 651–657.

Green, J. M. (1998): Differential tolerance of corn (*Zea mays*) inbreds to four sulfonylurea herbicides and bentazon. *Weed Technol.*, **12,** 474–477.

Green, J. M., Ulrich, J. F. (1993): Response of corn (*Zea mays*) inbreds and hybrids to sulfonylurea herbicides. *Weed Sci.*, **41,** 508–516.

Green, J. M., Ulrich, J. F. (1994): Response of maize (*Zea mays*) inbreds and hybrids to rimsulfuron. *Pestic. Sci.*, **40,** 187–191.

Kern, A.,D., Megitt, W. F., Penner, D. (1975): Influence of soil moisture on tolerance of corn to cyanazine. *Weed Sci.*, **23,** 522–524.

Miller, I. H. (1958): Differential response of several inbreds and single crosses of corn to 2,4-dichlorophenoxyacetic acid. *Diss. Abs.*, **18,** 1197–1198.

Penner, D. (1971): Effect of temperature on phytotoxicity and root uptake of several herbicides. *Weed Sci.*, **19,** 571–576.

Thomson, L. Jr., Slife, F. W., Butler, H. S. (1970): Environmental influence on the tolerance of corn to atrazine. *Weed Sci.*, **18,** 509–514.

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Kiss, G., Papp, I., Bakondi-Zámori, E., Gartner-Bánfalvi, Á. (1977): A szója fungicides magcsávázásának és rhizóbium oltásának együttes tanulmányozása. (Joint study of fungicide dressing and rhizobium inoculation in soybean.) *Növénytermelés,* **26,** 147–153.

Ouyang, J. (1986): Induction of pollen plants in *Triticum aestivum.* In: Hu, M., Yang, M. (eds), *Haploids of Higher Plants* ***in Vitro***. Academic Press, Beijing, pp. 26–41.

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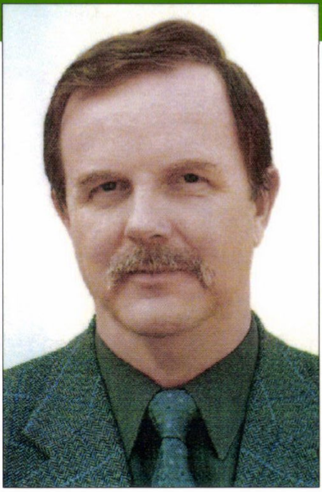

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